MORPHOGENESIS OF FILIFORM SPERM NUCLEI

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REVIEW OF LITERATURE

The study of morphogenesis of filiform sperm nuclei may contribute to our knowledge of the spatial relationship of individual chromosomes to each other.

One of the basic tenents of modern genetics is that chromosomes retain their identity and genetic information throughout the cell cycle.
The first cytological confirmation of this principle was given by Boveri (1887), and since then the evidence in favour of it was much extended (see for instance Davis, 1908; Hartmann, 1953 p.294-301; Schin, 1965; Pera und Schwarzacher, 1970). Not only in mitosis and meiosis is the individuality of the chromosomes retained but also during spermiogenesis.

Examination of the literature on positioning of individual chromosomes relative to each other in *elongate sperm(atid) nuclei* suggests that they may be randomly distributed (Mulsow, 1911; Wolf, 1939), aligned in parallel (Yuasa, 1937) or tandemly arranged (Hughes-Schrader, 1946, 1948, 1955; Inoué and Sato, 1962 a, 1962 b; Reitberger, 1964; Douglas, 1965; Costello, 1970 a, 1970 b; Moses and Wilson, 1970; see also Gerassimova, 1933; Dlugosz and Harrold, 1952; Taylor, 1964; Utakoji, 1966; Bianchi and Bianchi, 1969; Koehler, 1970). A critical review of the older literature was given by Tschermak-Woess (1963) and summarises date in plant (mosses, ferns) and animal species (worms, insects, mammalia) whose only common features are their possessing elongate or filiform sperm nuclei.

Although it is conceivable that chromosomal elements in *spherical sperm nuclei* could be positioned non-randomly (Comings, 1968; Comings and Okada, 1970), specific information for or against is not available primarily because of difficulty in determining relative positions of interphase chromosomes in these nuclei with aid of the light microscope. Electron microscopical techniques so far failed to detect individual chromosomes in most sperm nuclei (review of literature in Schin, 1965; and in Phillips, 1970) even when the arrangement of the chromosomes in a specific sperm nucleus was known from other sources (compare Hughes-Schrader, 1946 with Moses and Wilson, 1970). In fact, articals on electron microscopical features of spermiogenesis very hardly mention the word "chromosome".

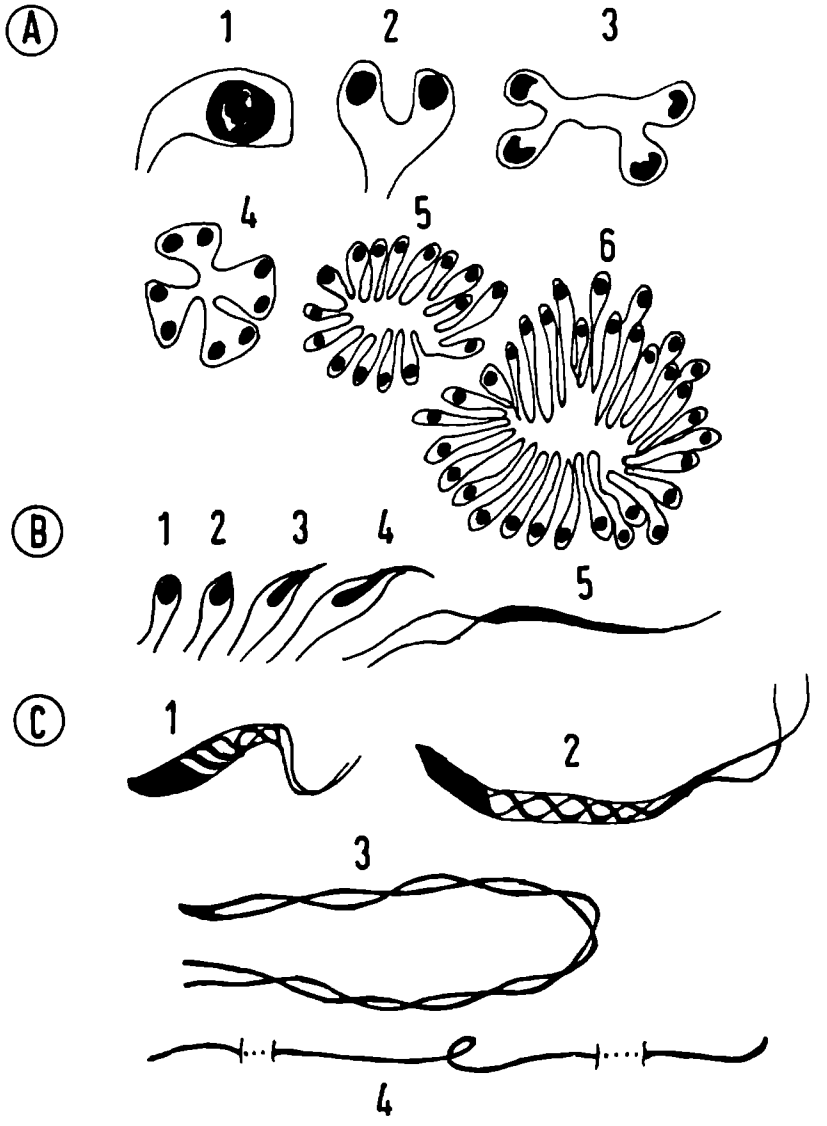

Fig.1 Crenobia - spermatogenesis - semi schematical (not at uniform scale)

A. Spermatogonium I (1) II (2) and III (3).
Spermatocyte I (4) and II (5);
Spermatid nuclei in syncytium (6).

B. Spermatid nucleus - sequence of nuclear elongation.

C. Spermatid nucleus - sequence of nuclear despiralization.

By means of the electron microscope positioning of individual chromosomes has been studied in *meiotic prophase nuclei* and for example the number and three dimensional interrelationships of synaptonemal complexes in these nuclei have received attention (Moens, 1969; Moens and Perkins, 1969).

In spermatid chromatin, DNA containing fibrils or lamellae might be expected to reflect the arrangement in mitotic or meiotic chromosomes (Schin, 1965). Careful study of spermatid nuclei with known arrangement of chromosomes may extend our knowledge of chromosomal fine structure and may even help solve the well-known controversy between interpretations of the chromosome as a unineme or polyneme structure (review of literature in Peveling, 1968; and Mulder, 1969; and Wolff, 1969; see also Heddle and Bodycote, 1969, 1970; Wolff, 1970; Laird, 1971). The parallel organization of DNA fibrils in elongate sperm nuclei is as difficult to explain in terms of a unineme as in terms of a polyneme chromosome model.

It has only recently become evident that spermiogenesis does not end with the formation of sperm cells. Rather more correct is to visualise meiosis, spermiogenesis, sperms transport and fertilization as subdivisions of a process. For example it is better to think of spermiogenesis as not being terminated in the testes but that sperm are still in preparation for fertilization after entering female storage organs (Jones, 1943; Makielski, 1966; Cantacuzène, 1968). It is during this last step that sperm capacitation occurs (Chang, 1951; Lakshman, 1971) although the specific changes in sperm while in receptacula have not been fully elucidated.

With this background in mind, the subject of this thesis was chosen to be a contribution to the cytology of the spermatid nucleus in animal species with filiform sperm nuclei, and the spatial distribution of the chromosomes within them.

PRESENT STUDIES

The morphogenesis of the sperm nucleus in the Plathelminth *Crenobia alpina*, as studied with light- and phase contrast microscopy, provides interesting clues concerning the transition from a spherical to elongate nucleus (Fig. 1). Elongating spermatid nuclei contain two plectonemically coiled, Feulgen-

positive threads, while only one long Feulgen-positive thread is found in the mature sperm (Linden, 1969 a). This sequence suggests hat this thread is folded on itself and twisted to form a plectonemic spiral in early spermatid nuclei. During spermiogenesis this spiral gradually uncoils to give the single filiform sperm nucleus. For verification of this highly unusual sequence of events, two different freshwater Plathelminth species were studied, *Dendrocoelum lacteum* and *Dugesia lugubris*, and spermiogenesis in these animals has been described using the same words as for *Crenobia* to accentuate the extreme similarity between species (Linden, 1970 a).

Elongating spermatid nuclei of Orthoptera are highly polymorph in appearance under standard experimental conditions; some nuclei show transverse banding strongly resembling individual polytene chromosomes (Linden, 1970 b). The latter observations prompted investigation of nuclear ultrastructure during spermiogenesis in *Schistocerca gregaria* (Orthoptera) with the electron microscope, using cut sections of preselected cells from squash preparations (for methods see Berendes and Meyer, 1968). However this research was not completed because the results did not add details to the general description of spermiogenesis in Orthoptera given by other authors (references in Phillips, 1970).

Unfixed Orthopteran spermatids were brought in contact with buffers of decreasing pH or alcohol-acetic acid fixative in a perfusion chamber, enabling one to observe cells before and during fixation. Using this technique it is seen that fixation or low pH cause contraction and may reduce nuclear volume to about 13% of the original. Also, irregular shapes or transverse banding of nuclear chromatin may result (Linden, 1971 a). The fibrillar and spiral type nuclei (see Linden, 1970 b) are not aberrant types because they are independent of fixation. The intermediate stages of elongating nuclei react more strongly than round or filiform ones.

An autoradiographic study was performed on *Schistocerca gregaria* chasing a pulse of radio-active tritium thymidine in meiotic chromosomes and spermatid nuclei. A variable number of chromosomes may be labeled at the first meiotic metaphase; a variable number of gaps is frequently found in elongated spermatid nuclei. These and other observations might be suggestive of tandem position of chromosomes in *Schistocerca* sperm nuclei (Linden, 1971 b), but the evidence is not conclusive. Autoradiographic evidence from sperm nuclei possibly cannot answer the question of chromosomal ordering, as long as the internal structure of chromosomes remains obscure.

To appreciate the properties of the spermatid nucleus, which is composed of chromosomes (more precisely chromatids) it seems essential to have a background knowledge of the mitotic and meiotic chromosomes. It is therefore appropriate to mention here more general chromosomal investigations that are not included in the thesis.

In a population of the Plathelminth *Crenobia alpina*, the level of polyploidy was determined by karyotyping (Linden, 1969 b). The level of ploidy in Plathelminthes is directly correlated with complicated pathways of gametogenesis sometimes observed (see for instance Benazzi Lentati, 1970). In this respect the results were meaningful to the later observations of spermatogenesis in the same *Crenobia* population (Linden, 1969 a).

Also connected with this thesis is the research on the fluorescence patterns of mice chromosomes (Hutten and Linden, 1971). This method enabled other researchers to detect the Y-chromosome in human sperm nuclei, by combining observations of metaphase chromosomes with those of sperm nuclei. The results suggest the possibility of determining the spatial distribution of chromosomes in filiform sperm nuclei with fluorescence microspectrophotometry.

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Spermatogenesis, Spermiogenesis and Sperm Structure in *Crenobia* (*Planaria*) *alpina* (Dana)

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Summary. Spermatogenesis in *Crenobia alpina* follows the general pattern found elsewhere in the Platyhelminthes, but spermiogenesis in this species reveals some unique features. The development of the spherical spermatid nucleus into the long, threadlike sperm nucleus is certainly not a simple elongation process as implied by earlier reports. Elongating spermatid nuclei contain two plectonemically coiled, Feulgen-positive threads, while only one long Feulgen-positive thread is found in the mature sperm. This sequence suggests that in early spermatid nuclei this thread is folded on itself and twisted to form a plectonemic spiral. During spermiogenesis it appears that this thread gradually uncoils to give the single elongated thread of the mature sperm.

The morphology and the dimensions of the mature sperms have been investigated and compared with earlier data. The early development of flagellae at the onset of spermiogenesis was not observed but some new sperm structures are described which are apparently involved in organising the twin flagellae. The precise structural organisation and functional significance of those structures is however not fully understood.

Introduction

The course of spermatogenesis in *Platyhelminthes* and the structural composition of their sperms is well known. The successive cell divisions of the spermatogonia are described for example by BENAZZI-LENTATI and NARDI (1950). Spermiogenesis is described simply as an elongation process of the nucleus and the sperm itself has an unelaborated structure (HYMAN, 1951, p. 118: NATH. 1965, p. 134).

For the present study attention was confined to the course of spermatogenesis and spermiogenesis in one population of *Crenobia alpina* (freshwater *Triclads*). *Crenobia* populations are interesting because they are regarded as relics which originated during the extinction of the Weichsel-ice-period (THIENEMANN, 1949, p. 35, quoted by DAHM, 1958, p. 81). The populations from different localities are completely isolated from each other.

The chromosome number of *Crenobia alpina* has been determined in a large number of populations, mainly by DAHM (1958); some degree of polyploid variation occurs both within and between populations. The chromosome number ($2n = 42$) and degree of ploidy of the present population has already been reported (LINDEN, 1969). The mode of reproduction in this population was entirely by sexual means (OOMEN and GEELEN, 1966).

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During the present study it appeared that within this population of *Crenobia alpina* spermatogenesis is of the conventional type for this group. However spermiogenesis and sperm architecture in this population reveal some entirely new and hitherto undescribed features.

Material and Methods

Samples were taken from the isolated population of *Crenobia alpina* from the brook in "het Filosofendal" on "de Duivelsberg" near Nijmegen (LINDEN, 1969). More than 100 specimen were screened during this investigation.

a) Squash Preparations. Squash preparations were made of the testicular region which lies anterior to the pharynx. Orcein 2% (in 60% acetic acid) stains fixed and unfixed material equally well. Fixation, whenever applied before orcein staining, was for two hours in alcohol: acetic acid (3:1).

Entire specimens (12 16 mm) were Feulgen stained (2 g basic fuchsine/100 ml) after fixation for two hours in fixative no. 3111 (alcohol:acetic acid: ethyl acetate: formalin 10% in proportions of 3:1:1:1) (VAN DER LINDEN) and hydrolysis for 5 min in N·HCl at 57° C. Background stain was removed with three changes of SO_2-water, 10 min each. Fixative no. 3111 preserved the bouquet-stages of the spermatocytes much better than the normal alcohol-acetic acid fixative. Also the stainability with Feulgen reagens was much improved.

Squashes of unstained material were observed with phase-contrast and dark field optics (Leitz condensor according to Heine) after fixation in no. 3111 for 5 hours.

b) Sperm Suspension. A suspension of sperms was obtained by disrupting the ducti deferens and bursae copulatrix of 5 animals in 5 ml 2% Na-citrate followed by cautious centrifuging (5 min at 500 RPM., radius of rotor 15 cm). The supernatant is discarded leaving $\pm$ 0,5 cc of fluid. Sperm preparations were made by pipetting a drop of the suspension onto a slide and forcing it to spread entirely. The slide is quickly immersed in the alcohol-ether (1:1) fixative and left there for 5 min. The sperms were stained in warm anilin blue — eosin B (GURR, 1965). Rinsing with distilled water removes the excess of stain. The slides were air-dried and mounted with euparal. Unstained sperms from the suspension were observed with phase-contrast. Photomicrographs were taken with the Leitz, Ortholux microscope, and camera on Agfa L Agepeff 35 mm film.

Observations

The classical outline of spermatogenesis in *Platyhelminthes* is as follows: 1) three generations spermatogonia (mitosis), 2) two generations spermatocytes (meiosis), 3) one generation spermatids (BENAZZI-LENTATI and NARDI, 1950). One original spermatogonium nucleus in *Crenobia*, gives rise to 32 spermatid nuclei through five successive and synchronous nuclear divisions. The nucleus of the original primary spermatogonium (Fig. 1) divides into 2 secundary spermatogonium nuclei (Fig. 2); through simultaneous divisions these give rise to 4 tertiary spermatogonium nuclei (Fig. 3), 8 primary spermatocyte nuclei, 16 secundary spermatocyte nuclei (Fig. 4), and 32 spermatid nuclei (Fig. 5).

This developmental sequence can be observed in *Crenobia alpina* very elegantly because no real cell division occurs. The nuclei which originate during the successive divisions remain within the original cytoplasm, whereby a syncytium is formed. Concluding each division the nuclei move periferically where they lodge themselves into finger-like protrusions of the central cytoplasm (Fig. 5). The cytoplasm increases in volume through each successive division (compare Figs. 1—5). Because of their appearance the syncytium thus formed is called a "bouquet" or "rosette".

The nuclei of the different stages of spermatogenesis usually appear in squash preparations either separately or in small groups, apparently because the cytoplasmic strands are easily disrupted. This can be remedied by using fixative no. 3111 (see Material and Methods).

Meiosis is normal. Mitotic spermatogonical stages were seen only infrequently but meiotic stages are more numerous. The most frequently seen meiotic stages were pachytene, diakinesis and metaphase I, while diplotene, anaphase stages and the entire meiosis II were rarely seen. This is an indication of the relative durations of these stages within the division cycle.

Spermiogenesis

The progress of spermiogenesis was judged entirely from morphological changes in the spermatid nucleus in orcein- or Feulgen-stained preparations. The formation of the flagellae and cell organelles escaped observation.

The spermatid nucleus that was formed as a result of the last spermatocyte division, is initially spherical, condensed, and optically amorphous (Fig. 5). This round nucleus gradually becomes pear-shaped, and the peripheral side develops a sharp point (Fig. 6). The nucleus elongates by an extension of this point to become cigar-shaped (Figs. 7, 8), and gradually the elongating nucleus starts sliding out of the bouquet cytoplasm.

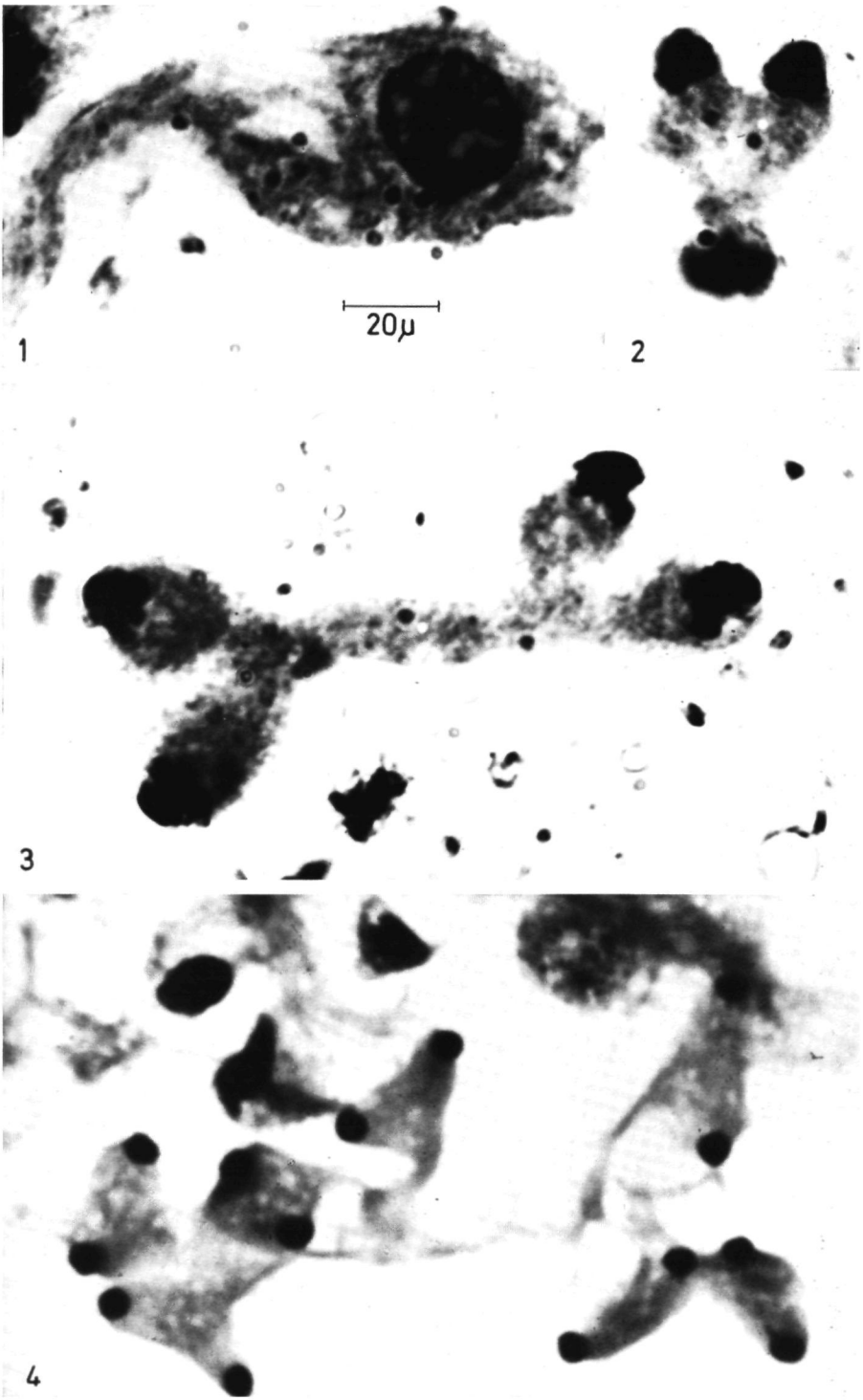
In Fig 9 the spermatids shown are almost free from the bouquet. It is in these stages that one can distinguish within the nucleus two separate, plectonemically spiralised, Feulgen positive threads (Figs. 10—12). Both dextral and sinistral spiralised forms occur in the same bouquet.

While the nuclei elongate, the coils of the spiral become more and more loosened (Fig. 13). In the mature sperms the nucleus consists of one single long, Feulgen-positive thread (Fig. 14), as was ascertained in Feulgen preparations of sperms from the ductus deferens. The diameter of this nucleus comes close to the diameter of one single spiral-thread in the spermatids. The mature sperm nucleus is more than twice as long as a spermatid nucleus from the stage of Fig. 9. From the combination of these observations we conclude that the two spiralised threads in the spermatid nucleus are in fact the parts of one single thread, folded on itself and subsequently twisted together. Thus spermiogenesis is partly the process of despiralisation of the spermatid nucleus resulting in the single threadlike sperm-nucleus. Stages where this process is almost completed, that is a spiral with only a few coils, were also seen in these preparations.

Mature Sperm

Feulgen and orcein staining of mature sperms from the ductus deferens and bursa copulatrix reveals only the filiform nucleus (Fig. 14); other sperm structures remain obscure.

Sperm taken from the suspension and stained with anilin blue — eosin B show the filiform sperm body; two equally long flagellae protrude sub-terminally from the sperm body (Fig. 15). This point where the flagellae leave the sperm, divides the sperm body into a short anterior part and a much longer posterior part (Table). The anterior part ends as a sharp needle. Whithin the sperm body,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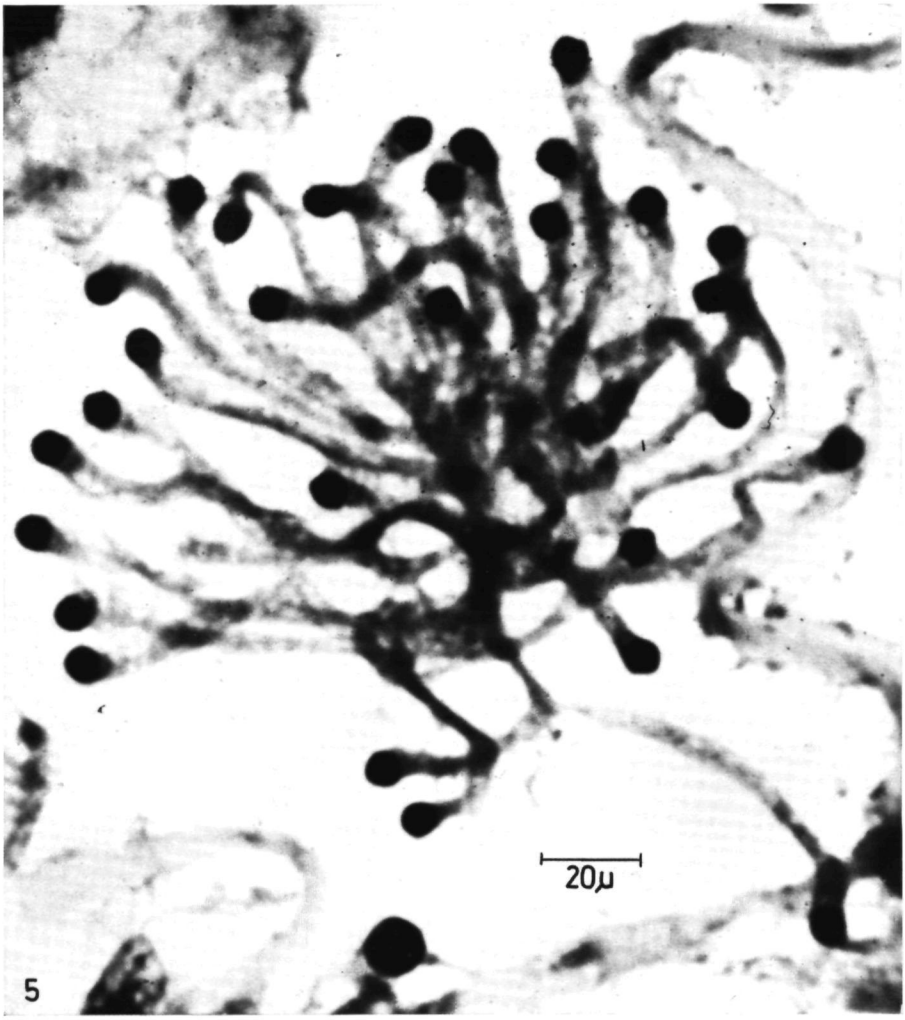
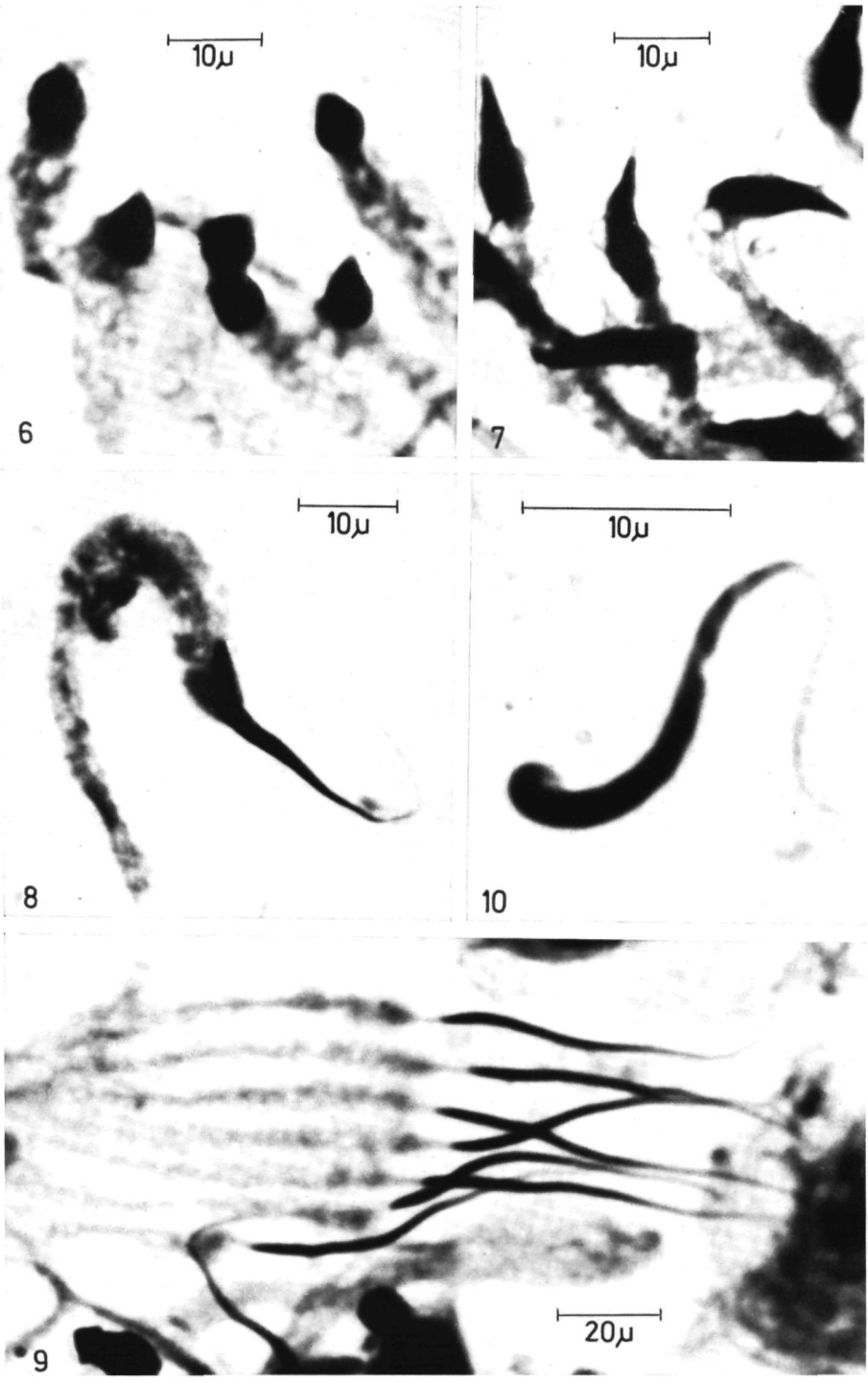


Fig. 5

Figs. 1—5. Spermatogenesis. Fig. 1. Primary. Fig. 2. Secondary. Fig. 3. Tertiary spermatogonium (*1*, *2* and *4* nuclei respectively). Fig. 4. Telophase of the division of 8 primary spermatocyte nuclei into 16 secondary spermatocyte nuclei (only 14 showing). Fig. 5. Bouquet of 32 spermatid nuclei (only 29 showing); orcein-stained preparations

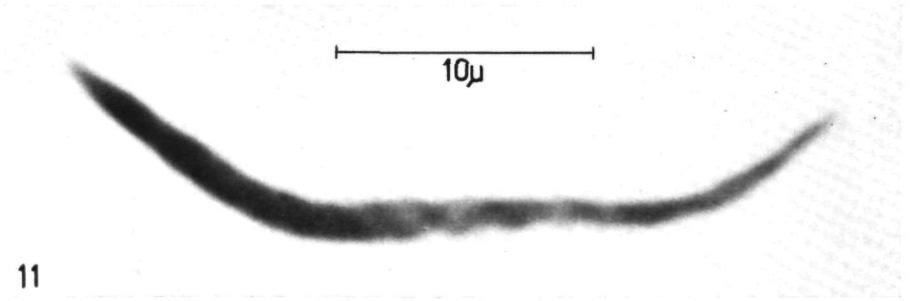
beginning behind the origin of the flagellae and extending posteriorly, there is a dense, slightly undulating, structure (Fig. 15a), evidently the filiform sperm nucleus, that is to be seen also in comparable squash preparations (Fig. 17).

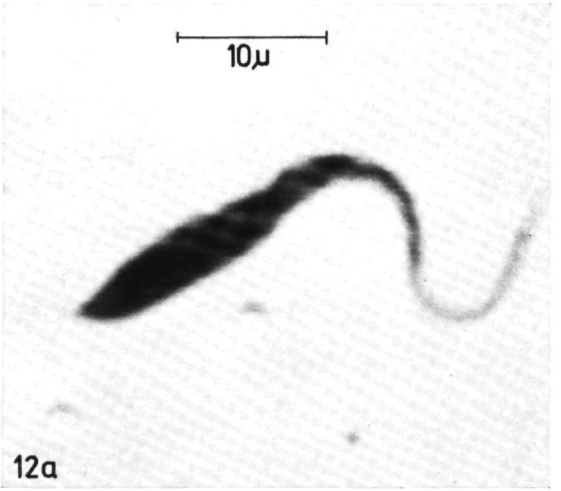
In unstained suspension preparations viewed with phase-contrast the morphology of the sperms appears different. The sperm body appears to be provided with ample cytoplasm, and within this cytoplasm a very thin, dark thread is discernable, running through the entire length of the posterior part of the sperm body and undulating slightly. The anterior end tapers out into a sharp needle

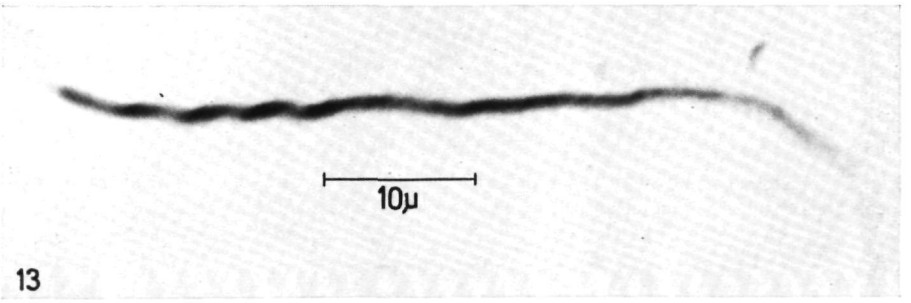


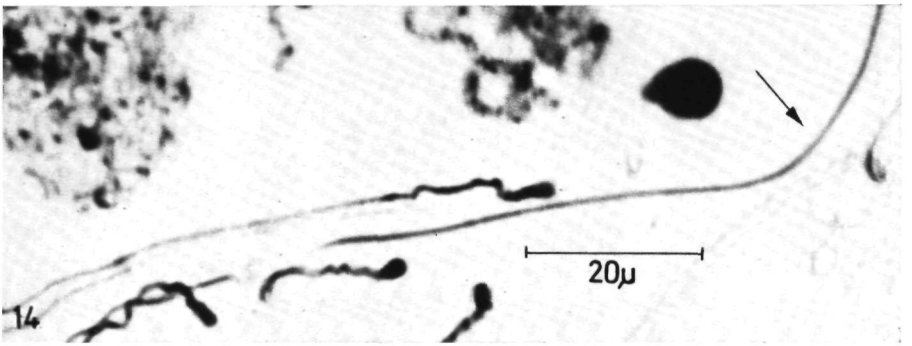
Figs. 6—10

Figs. 6—14. Spermiogenesis. Figs. 6—9. Progressive elongation of spermatid nuclei. Figs. 10—13. Spermatid nuclei corresponding to the developmental stages of Figs. 7—9, showing a double, plectonemic, spiral. Fig. 14. Parts of the, very long, mature sperm nuclei; orcein-stained preparations









Figs. 11—14

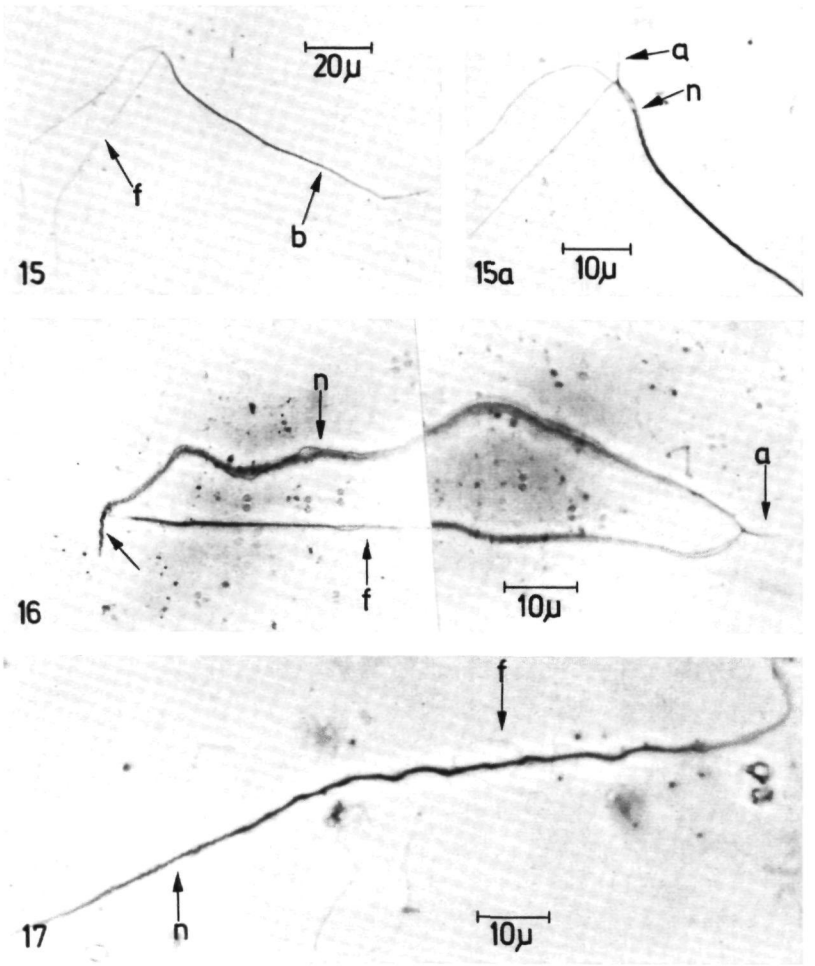


Fig. 15. Mature sperm from ductus deferens suspension (*b* sperm body; *f* flagellae); fixation alcohol-ether, aniline blue-eosine B stain. a The same sperm as Fig. 15; note the needleshaped anterior part of the sperm body (*a*) and the undulating nucleus (*n*)

Fig. 16. Mature sperm, type A, from ductus deferens suspension, the sperm nucleus (*n*) shows clearly within the abundant cytoplasm of the spermbody. In this sperm too the anterior part of the spermbody (*a*) is needle-shaped. The flagellae (*f*) are lying closely together. Fixation alcohol-ether, unstained, phase-contrast

Fig. 17. Immature sperm from a squash preparation. In the posterior part of the spermbody the nucleus (*n*) is seen to undulate as in Fig. 16: *f* is one of the flagellae. Fixative no. 3111, unstained, phase-contrast

(Fig. 16) as in the stained sperms. A synthesis of the observations from Feulgen and unstained suspension preparations suggests that the thin thread lying in the cytoplasm is the sperm nucleus.

The anterior part of one out of 9 measured sperms was very different (Fig. 18). Instead of being needle-like, this anterior part was blunt and contained much

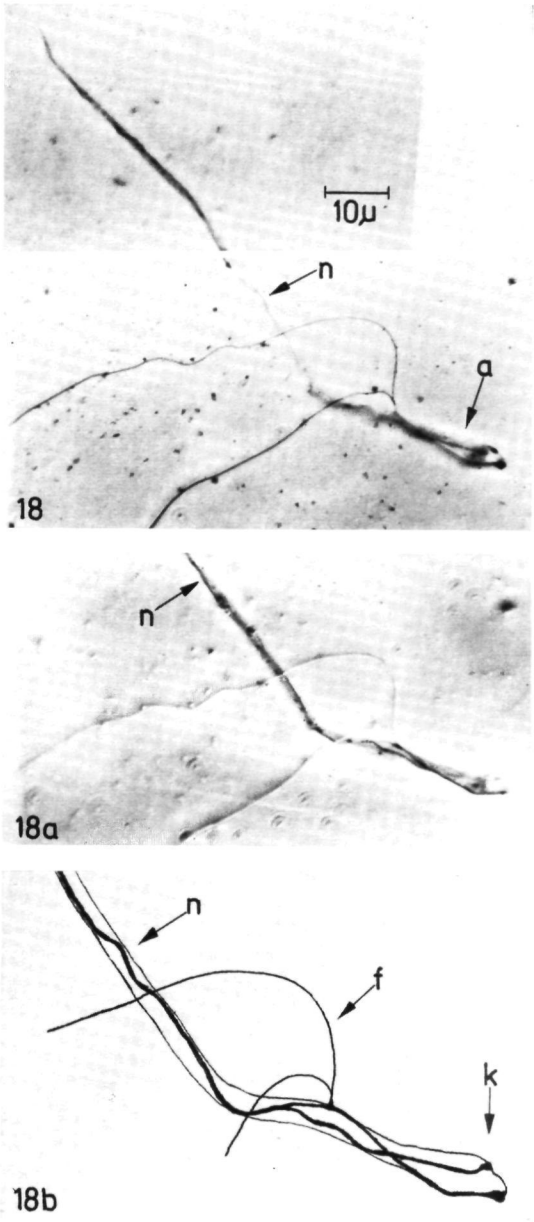
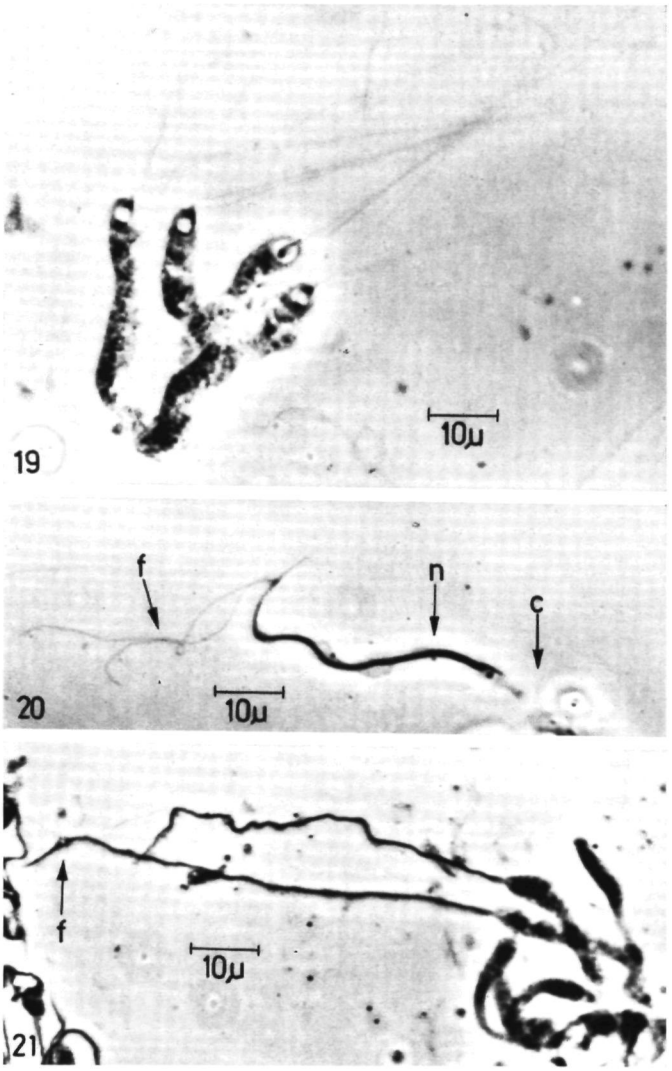


Fig. 18. a Mature sperm, type B, from ductus deferens suspension (see also the table, no. 1). (*a* anterior, *n* nucleus, *f* flagellae, *k* knob). The synthesis of these observations is given in b Fixation alcohol-ether, unstained, phase-contrast

cytoplasm. The single thread in the posterior end (the nucleus) appears to divide into two branches as soon as it enters the anterior part. Both threads end anteriorly near the cell membrane in a pair of dark knobs (Fig. 18a, b). The flagellae seem to have a common origin on one of the anterior threads.

The measured length of 9 sperms varied between 100—140 µ; the length of the flagellae varied between 70—115 µ, but they are always shorter than the



Figs. 19—21. Part of a spermatid-bouquet from a squash preparation. Fig. 19. Spermatids from the developmental stage of Fig. 6 show two thin threads protruding from the point of the nucleus, the flagellae. Fig. 20. Spermatid from the same stage as Fig. 9. The flagellae (*f*) are inserted subterminally in the spermbody; the anterior part of the sperm body is needle-shaped. The long dense nucleus (*n*) protrudes from the bouquet cytoplasm (*c*). Fig. 21. Spermatids in a very advanced stage of development (see also Fig. 17). The origin of the flagellae (*f*) is just visible; fixative no. 3111, unstained, phase-contrast

sperm body. The ratio posterior:anterior part of the sperm body is with exception of the first three sperms between 20—25. The ratio flagellum: sperm body lies between 0,80—0,99. There is no systematic difference between the measurements of phase-contrast and anilin blue — eosin B sperms (Table).

Table. *Dimension of sperm and flagellae of Crenobia, from a sperm-suspension sample from 5 animals. Fixation alcohol-ether (1:1) 5 min. No. 1—4, unstained, phase-contrast, No. 5—9, stained with anilin blue-eosin B*

The sperm body is divided into morphologically anterior and posterior parts by the origin of the flagellae.

a = anterior part of sperm body. p = posterior part of sperm body.

No.	Total sperm body length (μ)	a (μ)	p (μ)	Ratio p/a	Flagellae (μ)	Mean length (μ)	Ratio flagel/sperm body	
1	114	16	98	6.1	89— 96	93	0,82	phase contrast
2	102	7	95	13.5	85— 85	85	84	phase contrast
3	112	7	105	15.1	90— 90	90	80	phase contrast
4	127	6	121	20.2	99— 99	99	78	phase contrast
5	106	4	102	25.5	74— 74	74	70	anilinblue
6	103	4	99	24.8	101—101	101	99	anilinblue
7	116	5	111	22.2	95— 97	96	83	anilinblue
8	138	6	132	22 0	113—116	115	83	anilinblue
9	106	4	102	25.5	85— 85	85	80	anilinblue

Phase-Contrast Observations on Flagellae

In unstained preparations of spermatid bouquets, using phase-contrast, there are two very thin threads visible protruding from the bouquet-cytoplasm, even in the very early stages of spermatid nucleus elongation (Fig. 19). In later stages, when the spermatid nucleus is already very long, these two threads are seen to have a subterimnal joint attachment site on the spermatid (Figs. 20, 21). These threads are not visible in Feulgen preparations and are obviously the flagellae. Judging from their length, they are already fully developed in the earliest stage of spermatid nucleus elongation (Fig. 19). Comparing Figs. 8, 9, 20 and 21 shows that the anterior part of the sperm body goes in front when leaving the bouquet cytoplasm.

The shape of the nucleus in Fig. 20 matches that in Fig. 9; in Fig. 21 the nucleus has a uniform diameter over its entire length. These observations alone would lead to the wrong conclusion that spermiogenesis is a simple elongation process of the spermatid nucleus.

Discussion

Spermatogenesis

According to HYMAN (1951, p. 118, Fig. 41) meiosis I in *Turbellaria* takes place by a division of the primary spermatocytes. In *Crenobia alpina* this would be the division of the 8 spermatocyte nuclei. This could not be confirmed since diagnostic meiotic stages were not observed in an intact bouquet arrangement. However there is no reason why *Crenobia* should deviate from the scheme outlined by HYMAN.

Spermiogenesis

The metamorphosis of the nucleus during spermiogenesis, specifically the transformation of the double spiralled spermatid nucleus to the single threadlike

sperm nucleus, is so clear in Feulgen preparations, that evidently a change in the structural organisation of chromatin occurs between the spermatid stage and the mature sperm.

In a number of organisms which like *Crenobia* have long sperm nuclei, there is evidence for an ordered arrangement of chromosomes in mature sperm. Thus a tandem-arrangement of chromosomes has been demonstrated within the sperm nuclei of icerine coccids (HUGHES-SCHRADER, 1946), a liverwort (REITBERGER, 1964), grasshoppers (TAYLOR, 1964), *Drosophila* (COOPER, 1951), cave crickets (INOUÉ and SATO, 1962), and Chinese hamsters (DOUGLAS, 1965). Direct evidence on this point is lacking for *Crenobia*, but certain features of chromosome organisation at earlier development stages are compatible with a similar arrangement existing in this species. In particular spermatocyte stages show a strong polarisation of chromosomes which is regarded as a condition for later transformation to a tandem arrangement (DOUGLAS, 1965).

As mentioned earlier, both dextral and sinistral spiralisation of spermatid nuclei occurs. This observation suggests that these two forms originate in pairs and as mirror images of one another from the division of secondary spermatocytes in the way described for the liverwort *Sphaerocarpus* (REITBERGER, 1964). If this is the case then there should be equal numbers (16 each) of dextral and sinistral spiralised spermatid nuclei within one intact bouquet. We have not yet been able to test this hypothesis, nor the assumed tandem-arrangement of the chromosomes within the sperm nucleus.

Because the spiralisation of the spermatid nuclei disappears during the process of spermiogenesis, mature sperms developing from dextral and sinistral forms must be equivalent. Therefore the direction of spiralisation probably has no functional meaning.

Sperms

FRANZEN (1956) is very surprised to find that *Turbellaria* sperms possess more than one flagellum. He also observed that their sperms are usually long and possess ample cytoplasm. In these respects our findings in *Crenobia* are identical.

A discrepancy exists between the present measurements of sperm and flagellae lengths and those quoted elsewhere in the literature. According to RAPPEPORT (1915) (quoted by DAHM, 1958, p. 84), *Crenobia* sperms are 145—175 μ long, the two very thin flagellae are 65 μ long (fixation alcohol acetic acid 3·1. stain chrome haematoxilin after Gomori). The present measurements are 100—140 μ for length of sperms and 70—115 μ for the flagellae (fixation alcohol:ether 1:1: phase contrast or anilin blue-eosin B). These differences may be due to the different fixatives used, in which case we have the unlikely situation of a differential effect on the sperm body and the flagellae. Another possibility is that the discrepancies reflect a variation between the different populations sampled. There seems to be no systematical difference between the present measurements of phase-contrast and anilin blue-eosin B measurements (Table).

NATH (1965) observes that "neither the Golgi-bodies (or their derivatives) nor the mitochondria (or their derivatives) seem to be present" in *Turbellarian* sperm. A similar situation obtains in *Crenobia* (Figs. 15, 16, 18): in this material even the centrosomes from which the flagellae should originate seem to be absent.

However the results of SILVEIRA and PORTER (1965) dictate otherwise. Their electron microscope study of spermiogenesis in three Flatworm species, two marine and one freshwater (*Dugesia tigrina*), showed an identical spermatid development in these far related species. *Crenobia* is much closer related to *Dugesia* than the marine Flatworms. Because of the extreme uniformity between far related *Turbellarians*, described by SILVEIRA and PORTER (1965), it seems justifiable to extrapolate their results cautiously to *Crenobia*. What were described as the morphologically anterior and posterior ends of the sperm-body, appear in reality during movement of the sperms still to be anterior and posterior. This is an inversion of the usual situation in other animal sperms. The sperm body contains the filiform nucleus, starting near the point of flagellar attachment and extending posteriorly, together with one single mitochondrion alongside which extends and fills up the anterior region as well. The flagellae originate from 4 centrioles from which 2 are modified into basal bodies. The flagellae run a short length parallel to the long axis of the sperm body (which they call "sperm head" because they think an honest sperm should possess a mid-piece and tail as well; however the only structure that a sperm really needs is a nucleus!). The flagellae emerge on one side of the sperm body at the same level, and extend freely backwards for the rest of their length (SILVEIRA and PORTER, 1965, p. 254, Fig. 9). The anterior part of the sperm ends in a very sharp needle; SILVEIRA and PORTER (1965) have not been able to follow its origin through the early stages of spermatid development.

Returning to the present observations, one out of the nine measured sperms (Table, No. 1, and Figs. 18a, b) was different from the others: instead of being needle-shaped its anterior part was blunt and contained much cytoplasm. The single thread in the posterior end (the nucleus) appears to divide into two branches as soon as it enters the anterior part. Both branches of this thread end anteriorly near the cell membrane in one dark knob each. The flagellae seem to have a common origin on one of the anterior branches. It seems unlikely that this thread is a centrosome because a centrosome always appears to be situated on the same longitudinal axis as the flagellum, while in the present case it crosses the flagellum axis transversely. However, SILVEIRA and PORTER (1965), p. 242) do mention that the position of one centriole is oriented transversely to the long axis of the flagellum, in some other Flatworm species. Because of the difference in dimensions it seems improbable that these branches would be the equivalents of the transverse centriole, especially as they appear to extend from the nucleus. It is possible however that these threads do not fuse with the nucleus, but retain their individuality and coil around the nucleus. The undulations shown by the nucleus leave room for this speculation. Two tubuli which are found coiled underneath the outer membrane in sperms of *Cestodes* (RYBICKA, 1966) may represent comparable structures. The only difference is that in *Crenobia* these structures are closely apposed to the nucleus. Another possibility is that the two threads seen in *Crenobia* sperm are formed out of the micro-tubules which, according to SILVEIRA and PORTER (1965), lie just underneath the outer membrane in Flatworm sperms.

Two types of sperms have been described in this study, *type A* (Figs. 15, 16) with a needle-like anterior part and *type B* (Fig. 18) with a blunt anterior part and the two threads. The following possibilities must be considered:

1) type B is an aberration.

2) type A or type B is an artefact.

3) type A and type B represent different developmental stages.

4) both types occur normally in one specimen at the same time, and both are mature sperms.

ad 1) This can only be tested by observation of large samples taken separately from different animals. For instance it is possible that one of the five specimens from which the suspension was obtained, had aberrant sperms.

ad 2) Whether one of both types is an artefact must be tested by observation of unfixed sperms. It is worth noting however that the sperms shown in Fig. 16 (type A) and in Fig. 18 (type B) were on the same slide! Their treatment must therefore have been nearly identical.

ad 3) In this case we have to assume that the sperms develop further while in the ductus deferens. The second and third hypotheses assume a change in the length and volume of the anterior part of the sperm. The ratio posterior: anterior part of type B (Table, no. 1) is notably low. It is impossible to argue whether type A or type B is the most advanced in development.

ad 4) This hypothesis differs principally from the third by the assumption that both sperm types are mature. The difference between type A and B might originate from their physiological state.

Apparently it is quite common in *Planarians* for sperms to be morphologically different before and after ejaculation. Both forms can be found within the same animal, its own sperms in the ductus deferens and donor sperms from copulation, in its spermatheca. JONES (1943) describes a *Turbellarian* where the sperms are covered in a hyaline sheet which dissolves after copulation; this is to prevent self-fertilisation. Because our suspension was obtained from both the ductus deferens and the bursa copulatrix this explanation seems the most likely.

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Spermiogenesis in Flatworms is Not a Simple Process of Nuclear Elongation

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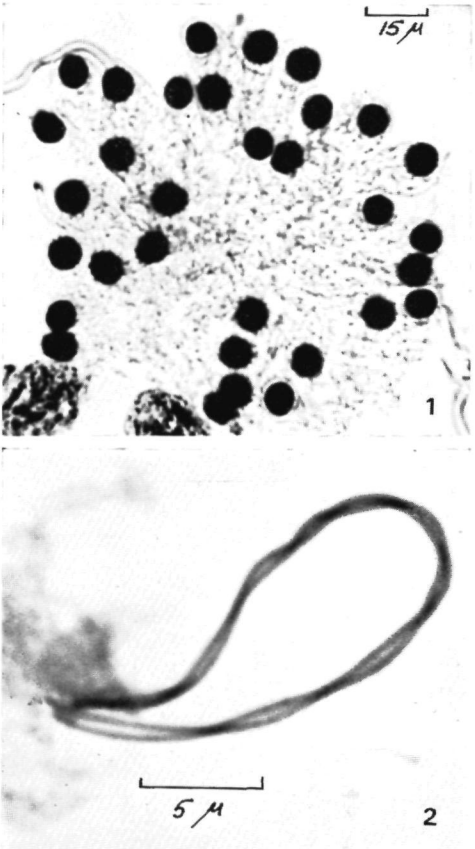
Specimens of *Dugesia lugubris* and *Dendrocoelum lacteum* were caught by hand in a lake on the grounds of the University of Birmingham. Five specimens of each species were fixed in No. 3111 (alcohol-ethyl acetate-acetic acid-formalin 10% in proportions of 3 : 1 : 1 : 1) (Linden 1969) for 4 hours. Hydrolysis of entire specimens in 5N HCl at room temperature (23°C) for 35 min was followed by staining with Feulgen reagens (pH 3.6) for four hours. Excess of stain was removed with three changes of SO_2 water, 10 min each. Squash preparations were made of the testicular region which lies anterior to the pharynx. The observations in both species are extremely similar, so that the following description applies to both. One original spermatogonial nucleus gives rise to 32 spermatid nuclei through five successive and synchronous nuclear divisions. No real cell division occurs. The nuclei which originate during the successive divisions remain within the original cytoplasm, whereby a syncitium is formed. Concluding each division the nuclei move peripherically where they lodge themselves into finger-like protrusions of the central cytoplasm. The spermatid nucleus that is formed as a result of the last spermatocyte division, is spherical, condensed, and optically amorphous (Fig. 1). This round nucleus becomes pear-shaped



Figs. 1-2. 1, *Dendrocoelum lacteum*. 32 spermatid nuclei in the common cytoplasm. 2, *Dendrocoelum lacteum*. Spermatid nucleus, showing two Feulgen-positive, plectonemically spiralised threads.

and the peripheral side develops a sharp point. The nucleus elongates by an extension of this point to become cigar-shaped and gradually the elongating nucleus starts sliding out of the cytoplasm.

It is in these stages that one can distinguish within the nucleus, two separate plectonemically spiralised Feulgen positive threads (Fig. 2). Both dextral and sinistral spiralised forms occur in the same syncitium. While the nuclei elongate, the coils of the spirals become more and more loosened, In the mature sperms the nucleus consists of one single long, Feulgen-positive thread. The diameter of this thread comes close to the diameter of one single spiral-thread in the spermatid nucleus. The mature sperm nucleus is more than twice as long as a spermatid nucleus from the stage of Fig. 2.

From the combination of these observations we conclude that the two spiralised threads in the spermatid nucleus are in fact the parts of one single thread, folded on itself and subsequently twisted together. Thus spermiogenesis is partly the process of despiralisation of the spermatid nucleus resulting in the single threadlike sperm nucleus.

Exactly the same observations were reported for spermiogenesis in another flatworm species, *Crenobia alpina* (Linden 1969a), where also a discussion of the implications of this unusual course of events has been given.

The present information shows an extreme uniformity in the course of spermiogenesis in these three flatworm species. An electron-microscope study of spermiogenesis in three different flatworm species, (one marine and two freshwater) showed the same degree of uniformity (Silveira and Porter 1965), which was there even more remarkable because the species were far related. The electron-microscope study was not concerned with the presently described structural changes and is therefore an independent source of information. It seems justified to expect that most flatworms will show the same described mode of spermiogenesis.

However we should be reluctant with any generalization because there is evidence of evolution and differentiation at various levels within the flatworms. In the species *Crenobia alpina* a beginning of speciation was reported. The particular population mentioned above, should be called a hexaploid when compared with other *Crenobia* populations. However it was found to be functionally diploid ($2n=42$), which means that the chromosomes could not be divided into six groups of 7 homologues but only into two groups of 21 homologous chromosomes (Linden 1969b). Furthermore it was thought that all flatworms uniformly possessed a $9+1$ pattern of microtubules in their flagellae. Recently it was shown that in some flatworm species there occurs a variation on this pattern (Costello *et al.* 1969).

The described morphological changes during spermiogenesis are apparently very complicated and need to be accurately controlled. This control is not likely to be located in the chromosomes themselves. In no other tissue, at any stage of the cell cycle, is the chromatin as condensed as in the spermatid

and sperm nucleus, and may therefore be regarded as genetically inactive. The control must be located in the cytoplasm of the syncitium which also synchronises the nuclear divisions (see also Giménez-Martín *et al.* 1968).

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Chromosomal Elements in Elongating Spermatid Nuclei of Grasshoppers

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Summary. Elongating spermatid nuclei of *Chorthippus brunneus*, *Ch. parallelus* and *Schistocerca gregaria* are characterised by many DNA-fibrils, roughly parallel to the long axis of the nucleus. Some of these nuclei show a transversal banding pattern which strongly resembles individual polytene chromosomes in somatic tissues of *Diptera* and plants. The morphological similarities, between banded spermatid nuclei and individual polytene chromosomes, suggest a parallel structure of identical sub-units in the spermatid nucleus.

Some other types of nuclear structure are described. All these types of elongating spermatid nuclei were found in natural populations of the three species.

A transversal banding pattern could be induced in fibrillar nuclei after treatment of testes in $ZnSO_4$.

Key-Words: Spermatids — Grasshoppers — Chromosomes.

Introduction

It is an inescapable supposition for modern genetics that chromosomes retain their identity throughout the cell cycle: this supposition has been established in a number of instances (Ref. in Schin, 1965, p. 481). Taylor (1964) was able to show, with autoradiographs of the grasshopper *Romalea microptera*, that in its elongated sperm nuclei, chromosomes still retain their individuality and form a tandem-arrangement with a random sequence of the chromosomes. For a number of plant and animal species with very long sperm nuclei, a similar tandem-arrangement of the chromosomes was observed (Ref. in Douglas, 1965; Linden, 1969). From this we may infer that the structure of such filiform sperm nuclei necessarily reflects the properties of individual chromosomes that make up this nucleus. Therefore, spontaneously occurring or induced changes in the morphology of spermatid nuclei, as in the present study, may lead to knowledge of the underlying structure of the chromosomes during this particular stage of their duplication-segregation cycle.

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Prof. Dr. S. J. Geerts and Dr. L. T. Douglas, Department of Genetics, University of Nijmegen, are acknowledged for critical reading and discussing the manuscript.

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Virus infections have been described to cause overcondensation and vacuolisation of chromatin (Diaz and Pavan, 1965 Brito da Cunha, Morgante, Pavan and Garrido, 1968) The possibility that the patterns of condensation and vacuolisation, described in the present study, were due to a virus infection was investigated

Materials and Methods

Samples were taken from two Birmingham populations of *Chorthippus brunneus*, during August 1968, 20 males from each population The testes were dissected in insect saline and the fat body removed The testes were then put into fresh fixative alcohol acetic acid 3 1, and stored for at least three weeks, to allow the material to harden Squash preparations were made breaking up a few follicles mechanically in a drop of lacto propionic orcein Seven *Schistocerca gregaria* males, from the Anti Locust Research Centre, London, were treated in exactly the same way

Testes from both species were hydrolized in 5 N HCl at room temperature (23° C) for 35 min followed by staining in Schiff reagent (pH 3 6) for four hours Background stain was removed by transferring through three changes of SO_2 water, 10 min each Feulgen stained follicles were squashed in a drop of 45% acetic acid

Two slides from two male *Ch parallelus*, from a New Forest population (near Southampton) were orcein stained Testes taken from eleven male *Sch gregaria* were divided into parts with roughly equal numbers of cysts and put into different solutions one part was immediately put into fresh fixative, the others were left for 10 min in insect saline, or 2% $CaCl_2$ or 2% $ZnSO_4$ or distilled water (table) After this treatment the parts of the testes were fixed separately and squashed in orcein as described above

An electron microscope observation was done on hemolymph of one male *Sch gregaria* whose sperm suggested a virus infection

Observations

In all three species, the spermatid nucleus immediately produced by the second meiotic division is spherical and stains heavily This nucleus elongates and passes through cigar and rod shaped stages to finally become the long and filiform sperm nucleus (ca $150 \times 0\,5\,\mu$) In the intermediate stages of elongation the nucleus shows a characteristic structure with many DNA fibrils, undulating, but roughly parallel to the long axis of the nucleus (Fig 1) Spermatid nuclei whose morphology differed strikingly from that described above were first seen in *Ch brunneus* Altogether eight types of elongating spermatid nuclei are described

1 The Fibrillar Type

In the elongating nucleus there are numerous DNA-fibrils, undulating, but roughly parallel to the long axis (Fig 1) This is the 'normal' type which is

Figs 1—8 *Chorthippus brunneus* Figs 9 and 10 *Schistocerca gregaria* (orcein, phase contrast)

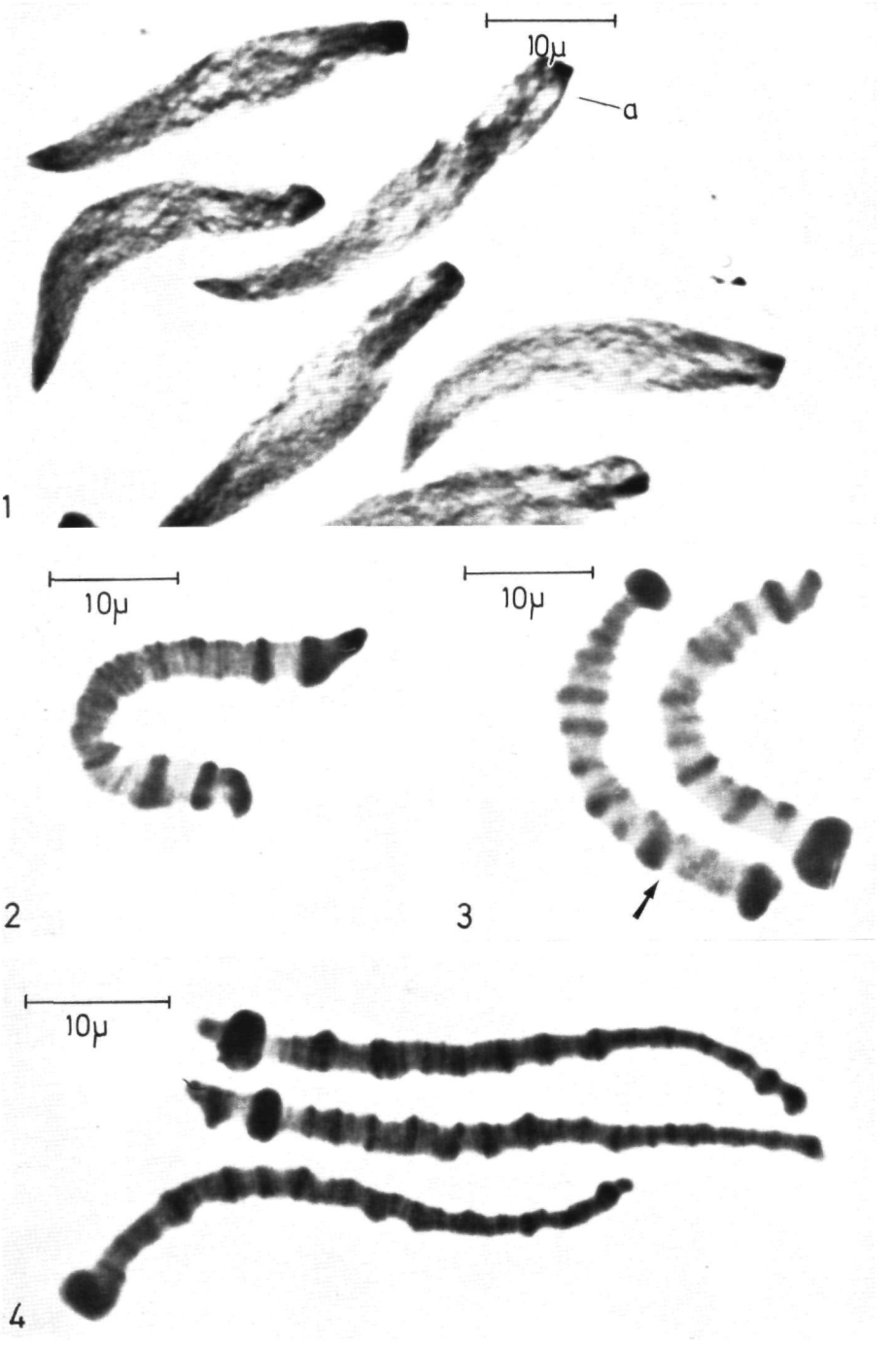
Fig 1 *The Fibrillar Type* Numerous DNA fibrils are longitudinally arranged in the spermatid nucleus

Figs 2—4 *The Banded Type*

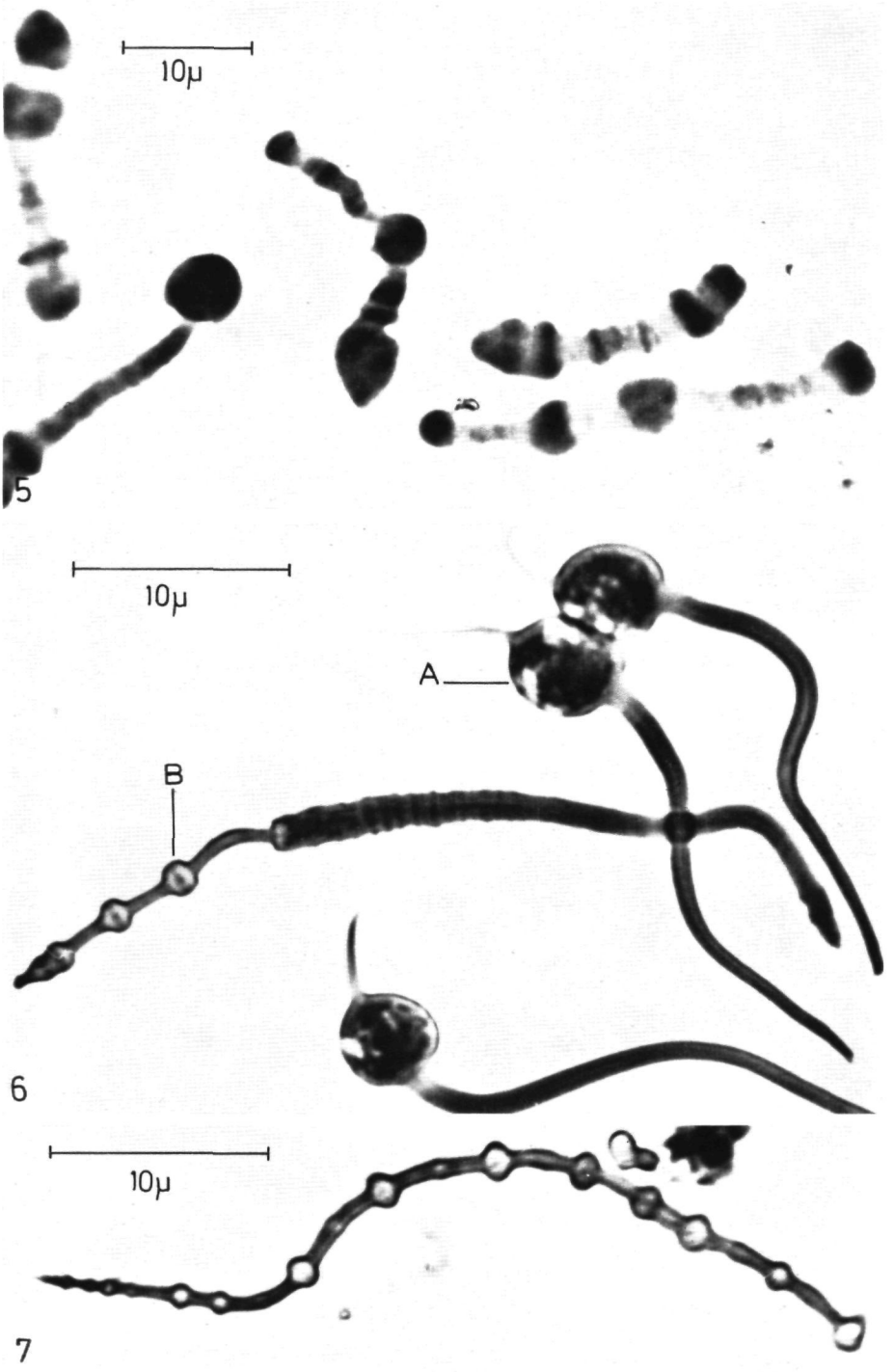
Fig 2 A number of very fine transversal bands is visible

Fig 3 Bands of greater size sometimes give the impression of a tight spiral of the entire nucleus (arrow)

Fig 4 Nuclei from the same cyst have the same appearance No regular sequence of bands was observed



Figs. 1—4



Figs. 5—7

the only one to be found in + 80% of all individuals In individuals which possess other nuclear types also, they still make up at least half of the types present The anterior end of the nucleus (Fig 1a) faces the tail of the sperm(atid) (Taylor, 1964)

2 The Banded Type

Along the length of the spermatid nucleus is an elaborated pattern of very fine transversal bands visible The size and number of bands shows a wide variation between nuclei The band size within nuclei is rather uniform (Figs 2—4) Some nuclei give the impression that the banding is the result of an extreme short spiral of the fibrillar bulk (Fig 3, arrow) A regular sequence of bands could not positively be identified This type of spermatid nucleus is more common than the other following types

3 The Stretched Type

Only parts of the nucleus have elongated Interspaced between those parts are portions of dense chromatin of irregular shapes and sizes (Fig 5)

4 The Globular Type

The originally spherical spermatid nucleus shows a bipolarity Both ends are drawn out from the main chromatin mass (Fig 6A)

5 The Vacuolized Type

The nucleus contains along its length a varying number of vacuoles of different sizes (Figs 6B, 7)

6 The Pointed Type

The ends of the nucleus are very thin and dense as in the mature sperm nucleus, the main body consists of irregularly dense chromatin (Fig 8)

These types of spermatid nuclei, except the *Vacuolized Type*, were later seen also in *Chorthippus parallelus* and *Schistocerca gregaria*

In addition there was found in *Sch gregaria*

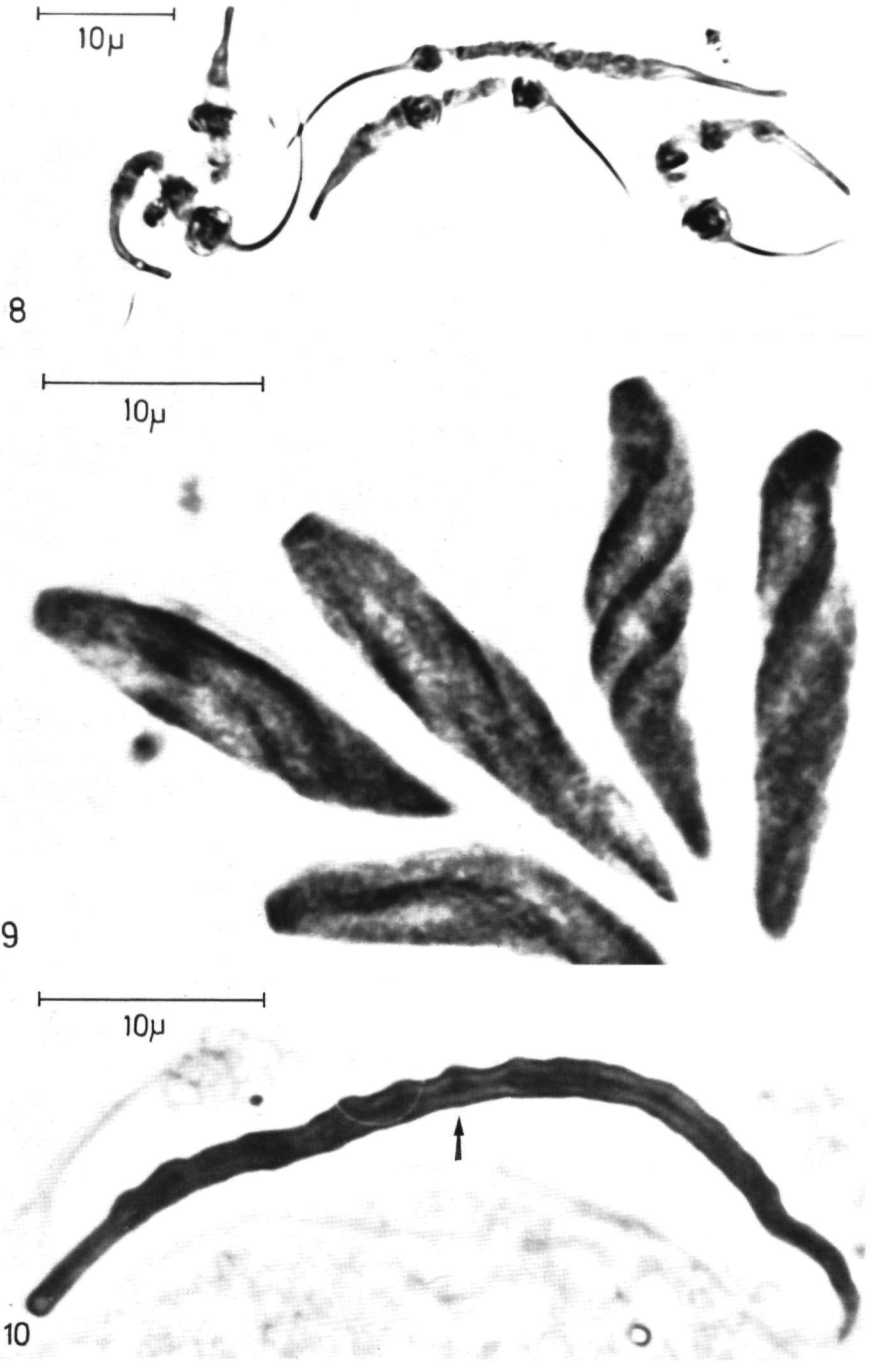
7 The Spiral Type

This is a basically fibrillar type nucleus, the fibrils join to form three or more ridges, which spiral around the long axis of the nucleus (Fig 9) This type is very common in *Sch gregaria* and might be called as ' normal' as the *Fibrillar Type*

Fig 5 *The Stretched Type* The chromatin mass is irregularly dispersed along the length of the nucleus

Fig 6 A *The Globular Type* The ends of the bipolar spherical spermatid nucleus, have elongated before the main chromatin mass started to do so B This nucleus shows the characteristics of the Banded Type (Fig 4) in the right part and of the Vacuolized Type (Fig 7) in the left part

Fig 7 *The Vacuolized Type* The spermatid nucleus contains a varying number of vacuoles along its length



Figs. 8—10

8. The Wave Type

Testes from *Schistocerca* with fibrillar spermatid nuclei only, were kept in a 2% $ZnSO_4$ solution prior to fixation (see also Material and Methods: table). In a number of nuclei the fibrillar appearance changed to a dense outlook: the outline of the nucleus undulates, each "wave"-top coincides with a transversal denser region (Fig. 10) (see also Discussion).

Table. *Schistocerca gregaria, treatment of testes. 11 males: A E are controls. 1—5 were injected for a different experiment (Hempa, a chemosterilant), which does not effect the present results. Testes dissected after 1, 9 or 11 days were divided into parts and put into the indicated ion-solutions. 10 min prior to fixation*

Specimen		Treatment				
		direct fixation	distilled water	2% $CaCl_2$	2% $ZnSO_4$	insect[a] saline
1 day	A	–	–			
	1	–				–
	2	–				–
9 days	B	±	–	–	+	–
	3	+				–
	4	–				–
11 days	C	–	–	–	–	
	D	–	–	–	+	
	E	–	–	–	±	
	5	–				–
	6	–				–

– means used treatment; + = after direct fixation, is the banding pattern found in natural populations, Figs. 2—4; – after Zinc-treatment is as in Fig. 10.

[a] Insect saline: NaCl 0.7 g; H_2O 100 cc; $CaCl_2$ 10% aq. 0.2 cc.

Testes from eleven *Sch. gregaria* were divided into parts and put into different solutions prior to fixation (Material and Methods, table). Only two testes showed banded spermatid nuclei (Fig. 4) after direct fixation. Follicles of the same testes, after 10 min in insect saline, did not show any banded nuclei; it was already noticed in *Chorthippus brunneus* that some follicles of the testes have "normal" fibrillar spermatid nuclei only. At the same time this feature does not effect the results after treatment of testes in 2% $ZnSO_4$. All four Zn-treated testes possessed a portion of Wave Type nuclei (table) which do not occur in natural populations. The above mentioned possibility of missing a certain nuclear type

Fig. 8. *The Pointed Type.* The only difference with the stretched type (Fig. 5) is that the ends of the nucleus are thin and dense as in the mature sperm nucleus

Fig. 9. *The Spiral Type* in *Schistocerca gregaria* is a Fibrillar Type; the fibrils form three or more ridges which spiral around the long axis of the spermatid nucleus

Fig. 10. *The Wave Type.* Fibrillar nuclei may become denser and show transversal dark bands at the same site where the outer boundary of the nucleus shows a "wave"-top, after treatment in $ZnSO_4$ (arrow)

in a given sample (for instance in insect saline) does therefore not apply. So it appears that Zinc-sulphate is capable of inducing transverse bands, in spermatid nuclei with a fibrillar structure. Osmotic pressure does not have a detected effect on the nuclear structure (table, distilled water versus different salt solutions). Also, the insect saline must be ruled out as a possible cause for the observed phenomena. The entire testes was checked in this experiment.

In the squash preparations there was no sight of virus crystals. Two slides were prepared for electron-microscopy of hemolymph from a male *Schistocerca* which possessed a proportion of banded spermatid nuclei in its testes. These preparations failed to show any sign of virus particles.

Discussion

All mature sperm nuclei are long, thin, heavily stained threads of chromatin. The vacuolized spermatid nucleus (Fig. 7) probably disintegrates: it is not known yet which ones of the other types develop into mature sperm nuclei.

Intermediate stages between the described nuclear types were observed, suggesting a developmental sequence. Short stretches of nuclei from the Banded, Stretched and Pointed Types (Figs. 3, 5, 8) still show indications of a fibrillar structure in the "inter band" regions and less dense parts. It seems therefore that these types have a basically fibrillar structure: by means of a localised contraction or condensation of these fibrils, or may be also by twists of the entire nucleus (compare Fig. 3 with Fig. 9), arises a pattern of bands or regions of condensed chromatin.

Fig. 6B shows a nucleus with a transversal banding pattern in one part and a vacuolised piece as the other part. This certainly represents an intermediate stage in the development from a Banded towards a Vacuolised Nucleus. A Globular Nucleus might develop into a Pointed one (compare Figs. 6A and 8). There is a great similarity between the Banded and Stretched Type (Figs. 3, 5) and between the Globular and Pointed Type (Figs. 6A, 8). They most likely form intermediate stages of two parallel developmental pathways (may be towards mature sperm nuclei). It appears that all spermatid nuclei from the same cyst not only belong to the same type, but also show the same degree of condensation (compare Figs. 3—5, 6A, 8, 9).

Two possible causes of the local condensation and vacuolisation of the chromatin material were considered. The first was that artefacts were produced by chemical compounds in the insect saline, used for dissection, whereas the second was, a possible infection of the animals with a virus or micro-organism. The results from the treatment of testes in different salt solutions (table) are only preliminary and show no effect of insect saline or change in osmotic pressure (distilled water versus high salt solutions, table): Zinc-ions induce a change in fibrillar spermatid nuclei resembling the banding pattern which occurs in natural populations (compare Fig. 10 and Figs. 2—4). It has to be shown that the Banded and Wave Type nuclei are not only morphologically but also structurally equivalent. The action of Zinc on fibrillar spermatid nuclei may be described to its ability to alter membrane permeabilities (Kroeger, 1967). It is not clear, in this stage, whether Zinc permits certain compounds from the nucleus to be washed

away or that it induces a true selective condensation of the fibrils in the nucleus. The important thing is that either way, it shows an identical response of the fibrils *in transverse sections* of the nucleus!

A suspected infection of the animals with virus or micro organism could not be substantiated. Slides of hemolymph from a male *Schistocerca*, which had a proportion of banded spermatid nuclei in its testes, failed to show any virus particles under the electron microscope. This does not necessarily mean that viruses are absent. It is possible that their concentration is very low.

There is a striking resemblance in morphology of the Banded Type spermatid nucleus (Fig. 4) and an individual Dipteran polytene chromosome. A structural resemblance exists between the synthetic Wave Type nucleus (Fig. 10) and polytene chromosomes in some plants. Polytene chromosomes in suspensor cells of *Phaseolus coccineus* have a structure of parallel fibrils (Nagl 1965, 1967); in *Phaseolus vulgaris* they have a granular appearance and by means of a cold-treatment it is possible to induce a very distinct transverse banding pattern (Nagl 1969). In both the present spermatid nuclei and in these plant polytene chromosomes there is initially a loose parallel structure in which an adequate treatment induces a pattern of transverse bands. It remains to be shown that the longitudinal DNA fibers in the spermatid nucleus have an identical response in transverse sections because the fiber parts involved in one band are *structurally* equal. In that case the consequences would include a polyneme structure of germ line chromosomes in the three grasshopper species.

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Short communication

Effect of pH on the morphology of elongating spermatid nuclei of grasshoppers

Der pH-Einfluß auf die Gestalt sich streckender Spermatiden-Kerne von Heuschrecken

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Abstract

Morphologically aberrant types of elongating spermatid nuclei in grasshoppers [4] are produced by the acid in alcohol-acetic acid fixative, contraction produced in Na-acetate buffer was more pronounced at low than at high *p*H's. Maximal contraction may reduce nuclear volume to about 13 % of the original and irregular shapes or transverse banding of the nuclear chromatin may result. Not all nuclei are equally susceptible to the acid and it is suggested that the degree of contraction observed is dependent on the developmental stage.

Introduction

In an earlier report [4] several classes of spermatid nuclei were described in three *Orthoptera* species, *Schistocerca gregaria, Chorthippus brunneus,* and *Ch. parallelus.* At that time two possible causes for the occurrence of aberrant types were suggested, one being that artifacts were attributable to chemical compounds in the insect saline, and the other virus infection; a third cause may be fixation, which will be shown responsible for the described aberrancies, which in itself is not unexpected. (The names for morphological classes of nuclei refer to the classification and figures given previously.)

Material and methods

Testes of *Schistocerca gregaria* and *Locusta migratoria* were studied (morphology of *Locusta* spermatid nuclei was very much the same as described for *Schistocerca). Schistocerca* males were obtained from the Department of Entomology, Wageningen. *Locusta* males were taken from the culture in the Department of Zoology, Nijmegen.

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[1]) Drs A. Van Der Linden, Department of Genetics, Driehuizerweg 200, Nijmegen, the Netherlands – The author thanks gratefully Prof. Dr S J Geerts, and Dr. L T Dougi as for discussions and for critically reading the manuscript.

The insect saline was 0.7 g NaCl, 0.2 ml CaCl (10 %), 100 ml H_2O and had *p*H 6.8. The Na-acetate buffers used were:
*p*H 5.84 (0.34 ml acetic acid + 7.38 g Na-acetate/100 ml)
*p*H 4.83 (0.44 ml acetic acid + 1.03 g Na acetate/100 ml)
*p*H 4.43 (0.70 ml acetic acid + 0.60 g Na acetate/100 ml)
*p*H 3.20 (5.37 ml acetic acid + 0.25 g Na acetate/100 ml)

Three fixatives were employed:
a) absolute alcohol-acetic acid 3 : 1 with *p*H 3.0
b) formalin 4 % with *p*H 7.0
c) glutaraldehyde 5 % in Na-cacodylate buffer *p*H 7.4

All *p*H's mentioned were measured. Orcein or Feulgen-stained squash preparations were screened for different classes of spermatids [4].

Fresh *Locusta* follicles were broken up mechanically in insect saline on a coverslip which was inverted and sealed with vaseline onto a split slide perfusion chamber [1]. Buffers were added with a pipette and the fluid drawn through the chamber by applying cotton to one side of the coverslip while the perfused fluid was added to the other. This method permits changing the fluid in the chamber within a second, without interrupting observations through the microscope. Macerated tissue was exposed successively to different buffers and allowed to react for a few minutes in each solution before being exposed to the next. This makes is possible to follow progressive change in any one nucleus, several of which were photographed at spread intervals through a Nomarski interference contrast microscope.

Results

Slides from three testes, each divided into two parts and fixed separately, were prepared according to Table 1. Formalin or glutaraldehyde fixed preparations exhibet spermatid nuclei only of the types previously classified as fibrillar and spiral [4], wheras material fixed in alcohol acetic acid frequently exhibits spermatid nuclei of the banded, stretched, globular, vacuolised and pointed type [4].

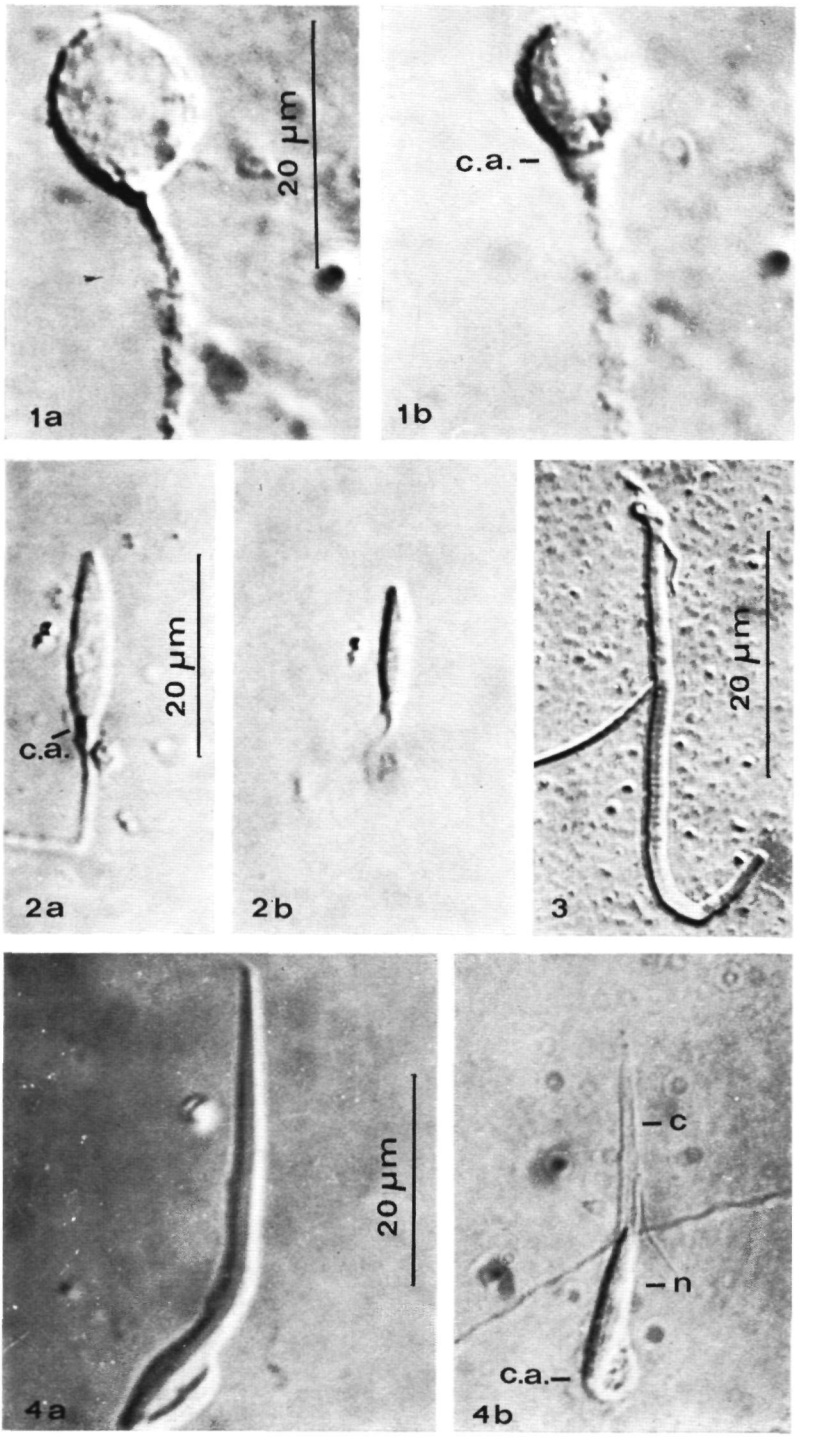
Table 1. Testes from three individuals divided in two parts, treated differently

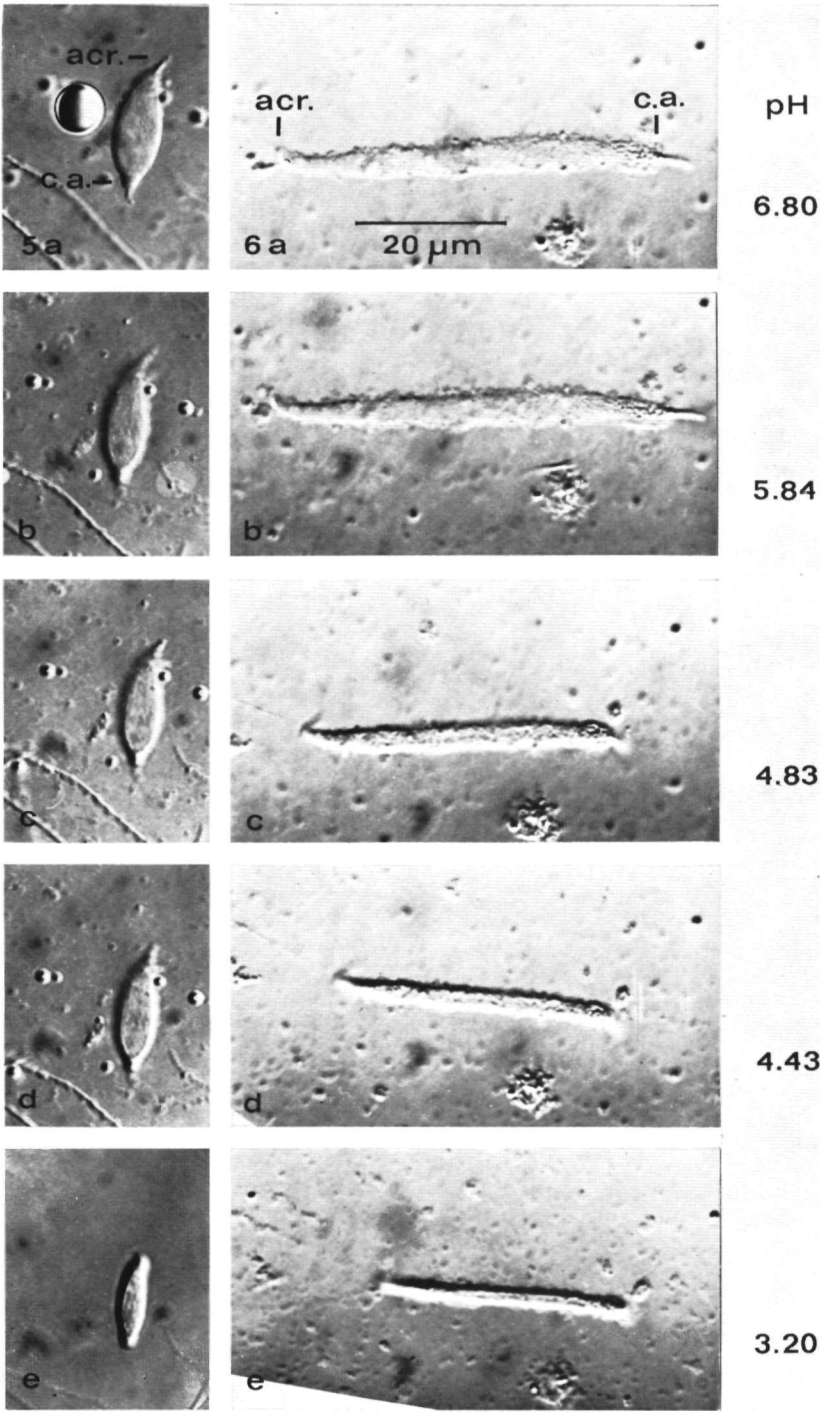
	Schistocerca				Locusta	
individual	I		II		III	
fixative	alcohol acetic	formalin	alcohol acetic	glutar-aldehyde	alcohol acetic	glutar-aldehyde
stain	orcein		Feulgen		Feulgen	

The volume of the nucleus decreases drastically with fixation (Fig. 1, 2, 4). Fig. 3 shows a fixed, elongated nucleus which evidently has a transverse banding structure, resulting from fixation. The nucleus shown in Fig. 4 a was observed and shrunk to its dimensions on Fig. 4 b within half a second.

To ascertain whether the nuclear contraction is caused by the alcohol or by the acetic acid in the fixative, one *Schistocerca* testes was divided into three parts which were fixed as follows:

Fig. 1 to 4. *Locusta migratoria* spermatid heads, unstained, Nomarski interference contrast – **Fig. 1 a, 2 a, 4 a** unfixed, insect saline – **Fig. 1 b, 2 b, 4 b** same cells after fixation with alcohol-acetic acid – **Fig. 3** nucleus with transverse banding after alcohol-acetic acid fixation – c cytoplasmic sheath – c. a. centriole adjunct – n nucleus


20 µm
1a
c.a.
1b
c.a.
20 µm
2a
2b
20 µm
3
20 µm
4a
c
n
c.a.
4b


acr.
c.a.
5a
acr.
c.a.
6a
20 µm
pH
6.80
b
b
5.84
c
c
4.83
d
d
4.43
e
e
3.20

a) immediatly in formalin

b) 0.5 min. in absolute alcohol, then changed to formalin

c) 0.5 min. in acetic acid, then changed to formalin.

After two hours fixation in formalin, the only preparations containing aberrant spermatid nuclei were from material prefixed in acetic acid.

In a perfusion chamber [1], acetate buffers of increasing acidity were brought in contact with unfixed spermatids. From Fig. 5 and 6 the effect is immediatly apparent: the volume of the sperm heads decreases at lower *p*H. From our earlier observations (phase contrast and electron microscopy, unpublished) is clear that the spermatid nucleus is surrounded by a very thin layer of cytoplasm; the nucleus extends the entire length of the sperm head from acrosome to centriole adjunct. Assuming that the nucleus is a cylinder, the decrease of nuclear volume can be computed from the figures. In Fig. 7 the volume of the spermatid nucleus from Fig. 6 is plotted against decreasing *p*H. At lower *p*H's the nuclear volume decreases rapidly and at *p*H 3.2 the nuclear volume is only 13 % of its original. Early spermatid nuclei (Fig. 1, 2, 5) and filiform sperm nuclei (not shown in figures) exhibit far less decrease in volume after alcohol-acetic acid fixation (roughly 30 to 70 %).

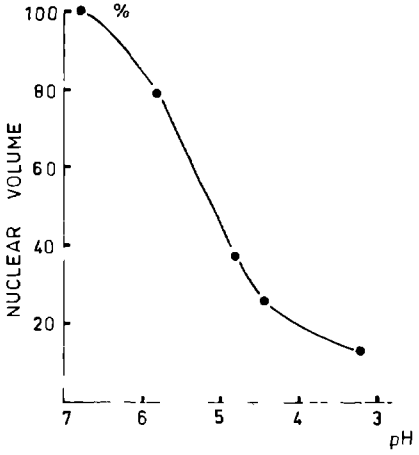


Fig. 7. The volume of the spermatid nucleus in percentages of the original, as computed from Fig. 6, in relation to *p*H of environment (Na-acetate buffers)

Discussion

The relation between nuclear volume and *p*H (Fig. 7) is S-shaped, as expected. The computation of volume was not attempted for the remaining figures as their shapes would produce too much error in the result.

Even though the production of aberrant nuclear types is evidently attributable exclusively to acetic acid, it is certain that the fibrillar and spiral type nuclei ([4] are not produced by fixation. In the intermediate stages of elongation, spermatid nuclei apparently react stronger to low *p*H than earlier and later stages which

Fig. 5 to 6. *Locusta migratoria* spermatid heads, unstained, Nomarski interference contrast The same two nuclei are shown at stages during perfusion with saline and Na-acetate buffers of decreasing *p*H – acr. acrosome – c a centriole adjunct

is probably correlated with the despiralization-contraction cycle of the DNA-containing material during spermiogenesis ([3] p. 162). In this respect it is tempting to speculate that the spiral type nucleus may have features in common with the spiral nucleus in muscle cells which may unwind and recontract, presumably in dependence of divalent ion concentrations [2].

Dark and light staining regions in young spermatid nuclei of the mosquito *Aedes aegypti* [5] resulted most likely from using acetic- or acetolactic orcein on unfixed cells.

Feulgen stained preparations show that contraction also (directly or indirectly) exerts an effect on DNA fibrils, producing dark and lighter regions on the nucleus. It may be that acidity has its primary effect on the protein framework within the nucleus and that the DNA fibrils which enclose this frame [6] move passively along with the contracting proteins.

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An autoradiographic study of elongating spermatid nuclei in Schistocerca gregaria (Forskal)

Autoradiographische Untersuchung über sich verlängernde Spermatiden-Kerne in Schistocerca gregaria (Forskal)

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Abstract

Morphological variation of elongating spermatid nuclei in *Schistocerca gregaria* [25] suggested investigation of the spatial arrangement of chromosomes in these nuclei.

Autoradiographic evidence from sperm(atid) nuclei of the grasshopper *Romalea microptera* [37] suggested that chromosomes are aligned in tandem along the nucleus of mature and nearly mature sperm.

The present results have similarities with Taylor's [37], but essentially they allow a critical evaluation of factors involved in this type of research.

Introduction

The allocyclic behaviour of the X-chromosome in male grasshoppers was described by Gerard [12] and Lewis and Robertson [23], and recently the X has been shown to be late-replicating [24] in *Schistocerca gregaria* also [14]; in *Romalea microptera* this property of the X enabled Taylor [37] to demonstrate tandem ordering of chromosomes in sperm nuclei.

In elongate sperm nuclei, chromosomes may be randomly distributed [31, 42], parallel aligned [44] or tandemly arranged [6, 7, 10, 16, 17, 18, 20, 21, 28, 34, 37; and perhaps also 9, 13, 22].

If it can be accepted that spermatid nuclei reflect the morphological properties of the chromosomes they contain [25], it would seem interesting to know whether a specific subsegment of the sperm(atid) nucleus is occupied by a specific chromosome; if this is known for *Schistocerca* the ocurrence of morphologically aberrant *spermatid* nuclei [25] dependent on *p*H [26] might be better understood. To determine the spatial organization of the chromosomes in sperm nuclei, labeling patterns of meiotic chromosomes and sperm(atid) nuclei of *Schistocerca gregaria* were studied after incorporation of tritium thymidine.

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Material and methods

Young male adult *Schistocerca gregaria,* from the Department of Entomology, Wageningen, were injected abdominally with one single dose of 15 uCi tritium thymidine in 0.03 ml sterile water, using a Hamilton 0 05 ml syringe (New England Nuclear, Frankfurt; specific activity 6.7 Ci/mM) Animals were kept in locust at 30° C and 12 hours light per day, and were sampled in pairs on day 1, 10, 12, 14, 20, 23, 31, and 34 after injection. Testes were dissected, the fat body removed under insect saline, and fixed separately in alcohol-acetic acid 3 1.

After a few weeks, follicles hydrolyzed in 5 N HCl 10 min. at room temperature, were stained in Feulgen 4 hours and washed in three changes of SO_2-water. Stained follicles were squashed in 45 % acetic acid between a slide and siliconized coverslip When placed vertically in 60 % acetic acid, the siliconized coverslip slides easily off (modified after Eberle, [11]) without much loss of material from the slide. The washed and dried slides were dipped in Kodak NB3 and taken out of the emulsion (45° C) very slowly to ensure a very thin layer. Dried slides stored at 4° C in light- tight boxes were developed, after seven weeks exposure, in Kodak D 19 at less than 15° C, fixed, dried and mounted in caedax.

Three preparations were screened for diagnostically labeled stages from each individual testes.

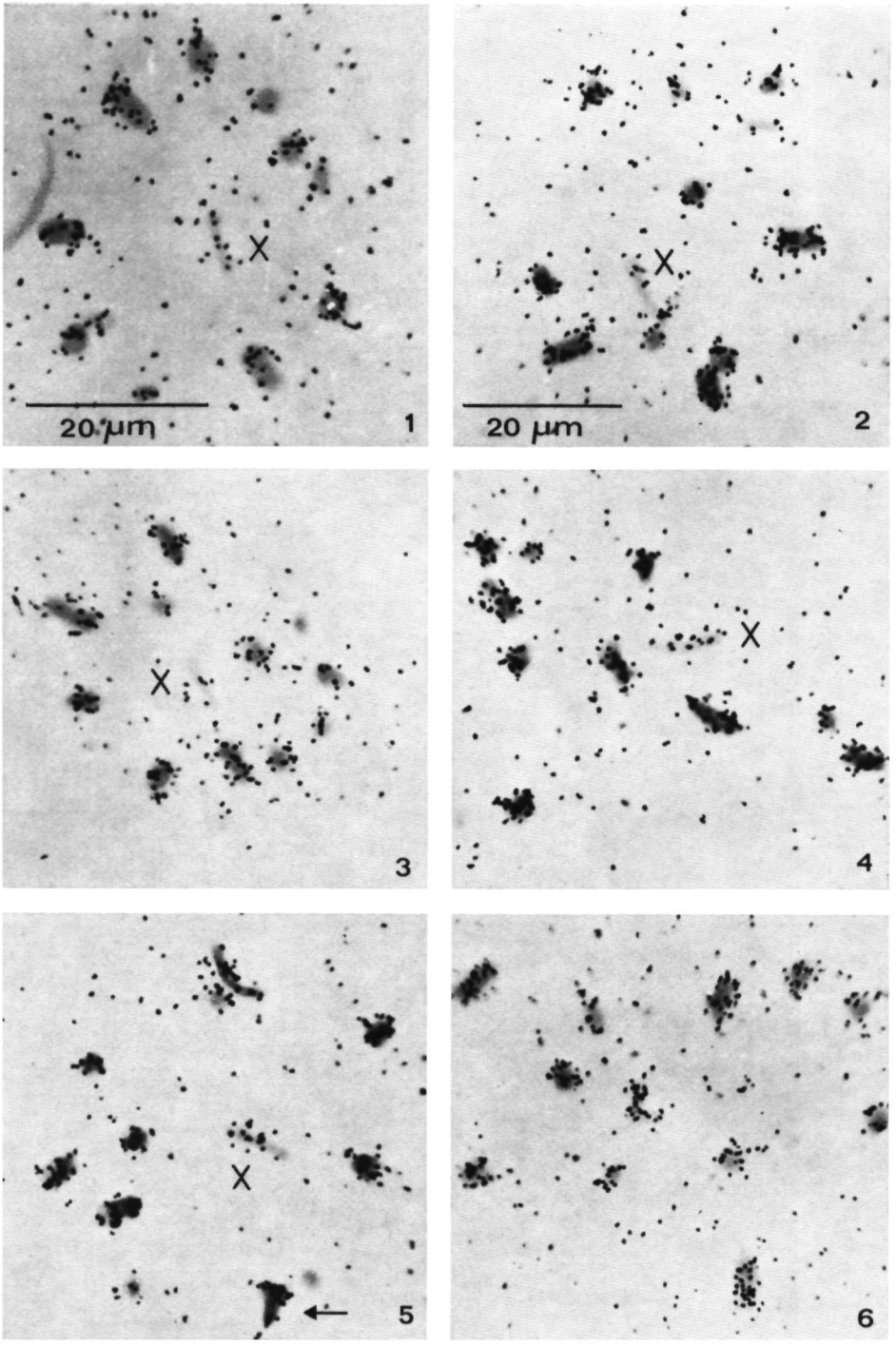
Results

The most advanced labeled stages at different periods (days) following injection are given in Table 1. On day 1 following injection, label in excess of the very slight background was not found. Pachytene labeling was observed at day 10, metaphase labeling on day 12, but after that (days 14 to 34) pachytene and metaphases I were never found labeled. In pachytene and metaphase I cells the X-chromosome has never been observed to be the only chromosome labeled. Different numbers of labeled

Table 1.

day after injection	most advanced labeled stage
1	—
10	pachytene
12	metaphase I
14	interstadia
20	elongating
23	spermatid
28	nuclei
28	filiform
31	sperm
34	nuclei

Fig. 1 to 6. Labeled metaphases I (X the univalent X-chromosome). The high background is limited to the area shown; background in the rest of these preparations was very low. Feulgen. Fig 2 to 6 at same magnification. – **Fig. 1.** 9 bivalents and the X labeled. – **Fig. 2.** 9 bivalents labeled. – **Fig. 3.** 8 bivalents labeled. – **Fig. 4.** 11 bivalents and the X labeled. – **Fig. 5.** 8 bivalents and the X partly labeled – **Fig. 6.** 11 bivalents and the X labeled (the X is not identifiable).


X
20 µm
1
X
20 µm
2
X
3
X
4
X
5
6

chromosomes per cell were found at metaphase I (Fig. 1 to 6). Also some chromosomes may be partly labeled (Fig. 5, arrow).

Rod shaped spermatid nuclei may be found labeled uniformly or they may exhibit a variable number of gaps (Fig. 7 to 10). The size of the label gaps varies within nuclei, and at later stages in elongation of the spermatid nucleus, the ease with which gaps can be distinguished and their number increases (Fig. 11 to 17).

The decision whether an observed gap really represents interrupted labeling was based on the following. Background label was subtracted from labeled nuclei; nuclear regions with label not exceeding the background were considered unlabeled.

Grain distribution exceeding the background does not extend farther than 1.5 μm away from the nuclear axis as measured in Figs. 11 to 17, so that under these experimental conditions, the maximum penetration of β-radiation is evidently 1.5 μm.

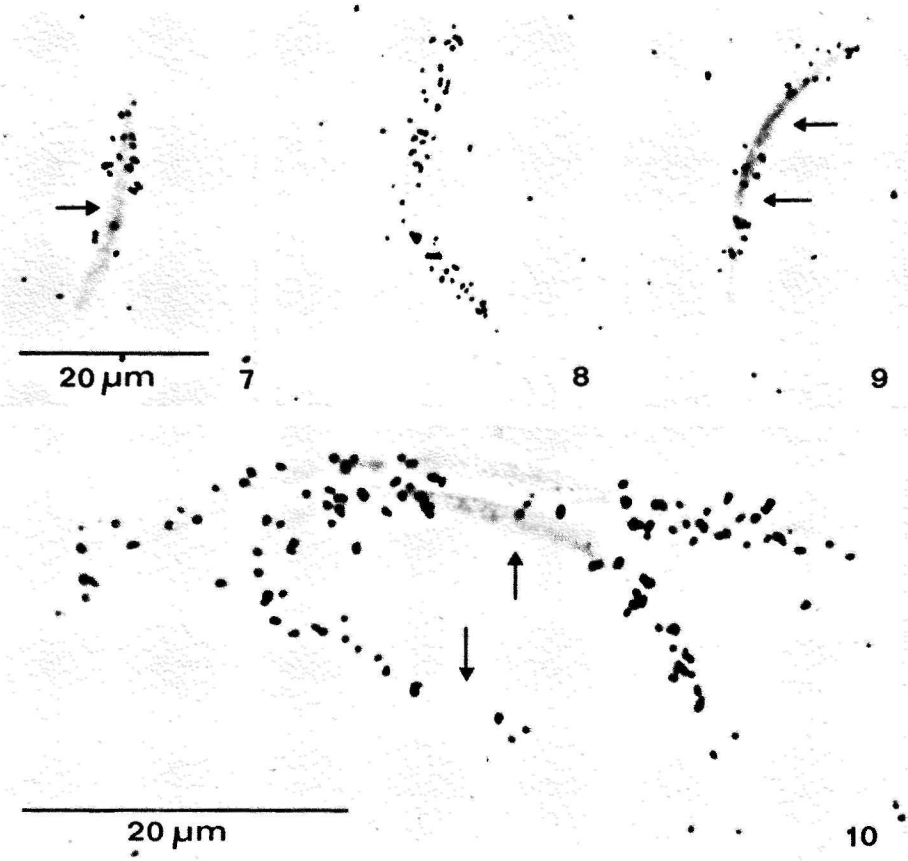


Fig. 7 to 10. Elongating spermatid nuclei, arrows point to label gaps. Feulgen. – **Fig. 7.** One gap or terminable labeled nucleus. – **Fig. 8.** In the middle portion of this nucleus a gap might be present but its length is less than 3 μm and therefore does not necessarily represent an interruption of tritium uptake. – **Fig. 9.** Two gaps and a terminal unlabeled region. – **Fig. 10.** Two nuclei, most likely from the same cyst, with a similar label pattern.

Therefore an unlabeled segment was taken to be a "gap" when no silver grains were present along twice this distance of 1.5 µm (3 um).When using such criteria, as many as five gaps greater than 3 um in length are positively identifiable in some nuclei. The diameter of the sperm(atid) nuclei on Fig. 11 to 17 is less than 0.5 µm and discontinuity in Feulgen staining was not observed.

Discussion

The maximum penetration of β-radiation in the emulsion used is known to be about 6 µm [1], although under the conditions here penetration farther than 1.5 µm was not detectable (see also [38] p. 208).

The time required to label mature sperm nuclei (28 days) under the present experimental conditions (rearing temperature 30° C) compares rather well with that reported for *Melanoplus differentialis* (22 days; rearing temperature 35° C, [29]), but not with data for *Romalea microptera* (60 days! culture temperature 23° C, [37]).

The duration of S-phase in *Schistocerca* is approximately one day [41]. One day after injection label was not incorporated at all. Thus the first detectable wave of labeled meiotic and of spermiogenic stages had undoubtably been incorporating tritium at the same time (pre- meiotic S-phase), which makes it possible to correlate labeling patterns in meiotic chromosomes with those in sperm nuclei (assuming that labeled nuclear regions indicate labeled chromosome regions). The failure of prophase meiocytes to incorporate label immediatly after pulsing suggests that uptake attributable to repair mechanisms [15] either did not occur or was not detectable and is consequently too weak to interfere with interpretations concerning S-phase synthesis.

According to BRYANT [3], displacement of label can occur in post S-stage chromatin when Feulgen-hydrolysis is carried out for 20 to 25 min. at 60° C in N HCl. In order to avoid such displacement, hydrolysis in the present study was shortened to 10 min. at 22° C in 5N HCl which is believed less severe and more easily to reproduce.

Only very few young spherical spermatid nuclei were found labeled, probably implying that the majority of these nuclei would have exhibited label prior to day 20 subsequent to injection (days 15 through 19 were not sampled). However these missing stages would probably not have permitted identification of individual chromosomes and therefore do not detract from conclusions based on elongate spermatid- and sperm nuclei.

More extensive observations on labeling of metaphase I chromosomes in male *Schistocerca*, corroborating the present data, are given by CRAIG-CAMERON [8]. In particular, this author observed that the X-chromosome is not the only one labeled in a given cell at any stage (see his Fig. 1). All possible numbers of labeled bivalents per cell were encountered, ranging from only one to eleven. Furthermore at the end of S-phase, individual chromosomes may be partly labeled either in euchromatic or heterochromatic segments.

Chromosomes are known to be arranged in a highly ordered manner in mitotic and meiotic interphases [27, 33, review of literature in 4] and in sperm nuclei (see introduction). Therefore, it seems justified to assume an ordered arrangement of chromosomes within *Schistocerca* sperm nuclei.

In a number of plant and animal species, chromosomes are known to attach end-to-end during mitotic interphase [2, 36, 39] and also during meiotic prophase [19, 40].

A DNA-positive interconnection between two tandemly arranged chromosomes was observed in the sperm of icerine coccids [16, 17, 28]. This evidence suggests the hypothesis that chromosomes associate intimately in tandem in all interphase nuclei, not only the sperm nucleus. It may even be that telomeres attach to the nuclear membrane in a modified type of tandem association. From this point of view, it is quite conceivable that no Feulgen-negative gaps separating individual chromosomes in sperm nuclei were seen in Taylor's [37] and the present study. Whether or not the interconnection between chromosomes may sometimes be Feulgen-negative is not clear (see [34]).

The conceivable modes of chromosomal ordering in elongate sperm nuclei are tandem or parallel. Alternatively, the chromosomes may be partially parallel. It has

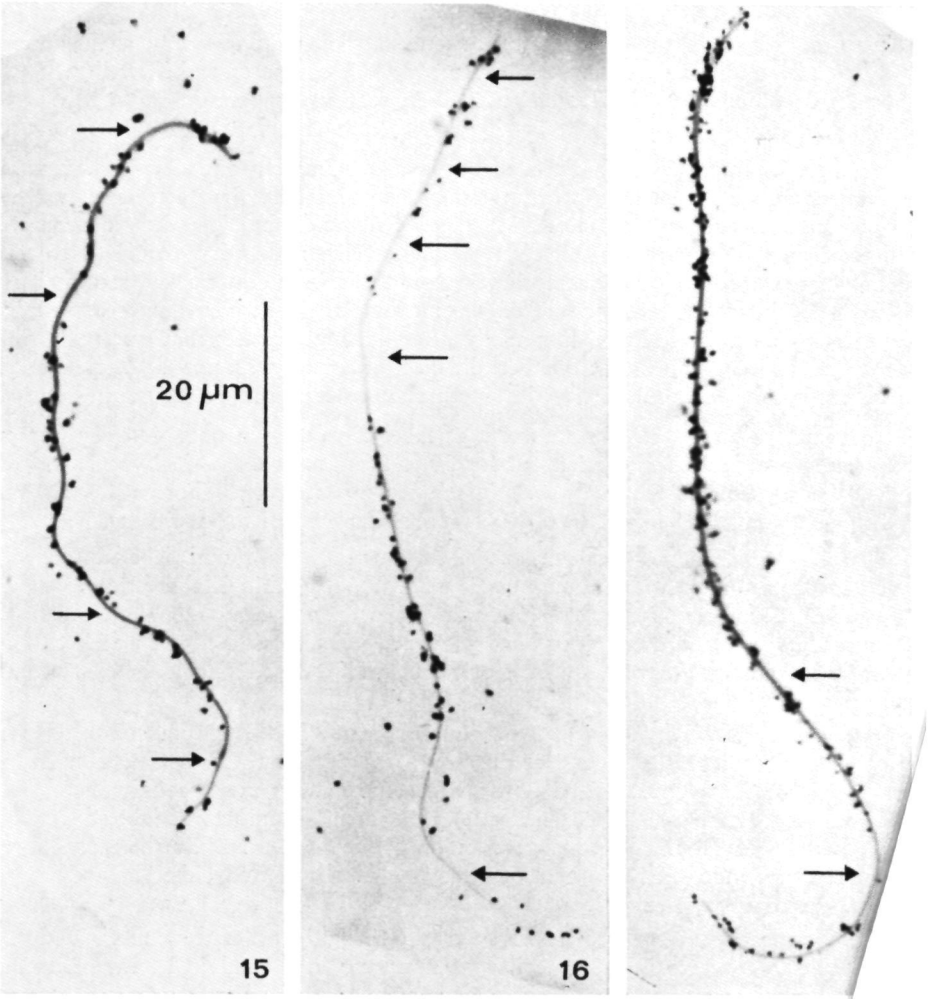
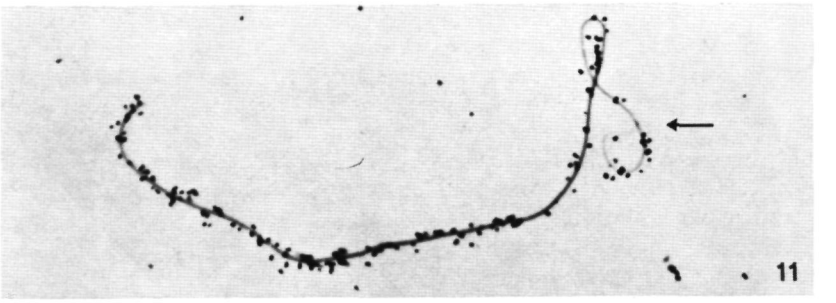


Fig. 11 to 17. Labeled filiform sperm (atid) nuclei. Arrows point to gaps. Feulgen. – One gap is positively identified. – **Fig. 12.** Three gaps indicated. The middle and arrow point to regions with *at least* one gap. – **Fig. 13.** Both terminal regions of th are unlabeled. – **Fig. 14.** Five gaps and a terminal unlabeled region present. – **Fi** gaps. – **Fig. 16.** Five gaps. – **Fig. 17.** Two gaps.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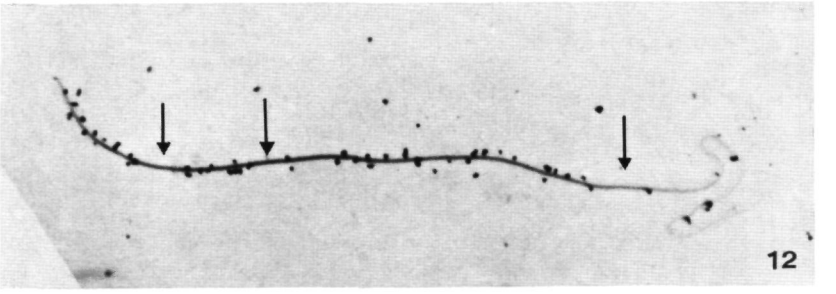
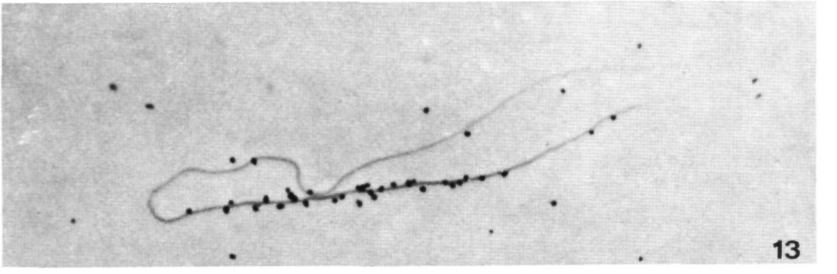
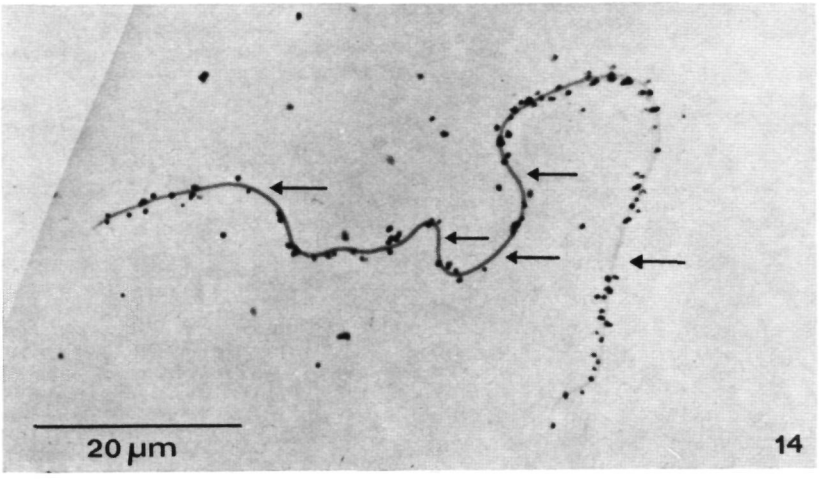

12


13


20 µm
14

been suggested that chromosomes in spermatid nuclei of *Romalea,* starting with parallel ordering, would slide past each other as elongation of the nucleus gradually eliminates overlapping to give eventually tandem alignment [37]; so tandem arrangement might be considered to be the extreme form of partial parallel alignment. The parallel organization of DNA fibers longitudinally in the sperm nucleus ([25], fig. 1, references in [35, 43]) poses an equally difficult problem for understanding parallel or tandem alignment of chromosomes; the solution of this problem is directly related to understanding of chromosome structure itself.

Another interesting difference between tandem and parallel alignment relates to geometric packing of chromosomes in the nucleus. Reaching tandem alignment in the fully ripe sperm nucleus (80 to 100 um) would require only a slight elongation of the individual chromosomes; whereas transition to parallel alignment would require greater than tenfolg stretching. Such stretching is comparable to reverting back to the interphase uncoiled state ([17] p. 161).

Twelve leptotene-size threads would probably not fit in a ripe sperm nucleus with parallel alignment of threads. The diameter of sperm nuclei (fig. 11 to 17) is less than 0.5 um and leptotene chromatids of *Schistocerca* are slightly less than 0.2 µm in diameter, so that it is difficult to conceive more than 6 or 7 such parallel threads being packed.

Given the observation that a variable number of chromosomes per nucleus may be labeled at metaphase I, one would expect to find sperm nuclei almost uniformly labeled or unlabeled, indicating parallel alignment of chromosomes. Alternatively, sperm nuclei might be found with a variable number of gaps in label suggesting a tandem arrangement of chromosomes. The situation is complicated by the possibility of partial overlap of chromosomes, and by the asynchronous behaviour of eu- and heterochromatic regions at the end of S-phase [8]. Some chromosomes may exhibit one or two terminally labeled regions.

Another difficult point is that present day knowledge of chromosome structure and behaviour is still too fragmentary to predict what happens on the submicroscopic level with incorporated tritium distribution in stretched chromosomes. For example it is theoretically possible that a uniformly labeled metaphase chromosome exhibits non – uniform label (gaps) after uncoiling (see for example [32, 5]); on the other hand, it seems that during the S-period many or perhaps all of the replicating units, in a salivary-gland nucleus of *Drosophila,* start DNA synthesis simultaneously but complete it at different times [30]. So if it may be assumed that a uniformly labeled metaphase chromosome remains uniformly labeled after uncoiling, a tandem alignment of chromosomes in sperm is indicated.

For example, when a parallel alignment is considered and only one chromosome per sperm nucleus is labeled, and partly labeled as well, one labeled spot will be found per nucleus. When two chromosomes are partly labeled, one or two regions per sperm nucleus may be labeled (one gap may be present) dependent of whether or not the labeled regions overlap. Data suggest that when three or more chromosomes are labeled, at least one of those is expected to be labeled uniformly during metaphase (compare with [8], Table 1) so the sperm nucleus would be labeled entirely. More than one gap in the label of sperm nuclei would not be expected.

When 11 or 12 uncoiled chromosomes are parallel or partial parallel in the sperm nucleus then, just by chance, label will be uniformly distributed. Mainly when few chromosomes per cell are labeled will there be a chance of producing gaps in a statistically unsignificant number of sperm nuclei. In the present investigation spermatid

nuclei with two to five gaps were frequently observed. The best explanation to fit these observations is that chromosomes are tandemly arranged within the sperm nucleus of *Schistocerca gregaria*.

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CURRICULUM VITAE

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Canisius College te Nijmegen, 1955-1960, diploma HBS-B; van 1960-1961 o.a. jongen aan dek, Koninklijke Nederlandse Stoomboot-Maatschappij, Amsterdam en leerling analist, kunstzijde spinnerij Nyma, Nijmegen. Ingeschreven als student biologie te Nijmegen in 1961. Kandidaats examen biologie 7-7-1965. In 1966-1967, 6 maanden stage medische etmologie op het Lab. voor Parasitologie, Instituut voor Tropische Hygiene/Universiteit van Amsterdam.
Doctoraal examen biologie, cum laude, 6-12-1967, hoofdvak zoologie met botanie en genetica. Van 6-12-1967 tot 1-10-1971 wetenschappelijk medewerker op de afdeling Genetica (Directeur Prof. Dr. S.J. Geerts) van de universiteit te Nijmegen. Van juli 1968 tot juni 1969 Research Fellow van het Department of Genetics, University of Birmingham, Engeland. Na terugkeer uit Engeland werd mij de leiding van de cursus Cytogenetica opgedragen.
Op 1-1-1970 werd ik aangesteld als wetenschappelijk medewerker in de rang van hoofd assistent en op 1-7-1971 volgde bevordering tot wetenschappelijk ambtenaar eerste klasse.

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Van degenen die aan het gereedkomen van dit proefschrift hebben bijgedragen wil ik speciaal Dr. L.T. Douglas hartelijk danken voor de vele enthousiaste lessen en de toewijding en bereidwilligheid waarmee hij aan ieder verzoek om hulp tegemoet kwam.
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STELLINGEN

I

De draadvormige kern in spermatozoiden van Plathelminthes ontstaat door despiralisatie uit de dubbelgevouwen en plectonemisch gewonden spermatide nucleus.

Dit proefschrift.

II

In een azijnzuurhoudend fixatief met lage pH, kunnen spermatide nuclei van Orthoptera plots sterk in volume afnemen waarbij morfologisch van elkaar verschillende typen kernen ontstaan.

Dit proefschrift.

III

De als "fibrillar- and spiral type" gedefinieerde spermatide nuclei van Orthoptera zijn geen fixatie artefacten.

Dit proefschrift.

IV

Zolang de inwendige chromosoom structuur onopgehelderd blijft is het niet mogelijk om uit autoradiogrammen van spermatozoiden tot de ruimtelijke ordening van de chromosomen binnen de kern te besluiten.

Dit proefschrift.

V

Een eventuele "biologen-eed" zou niet tot biologen beperkt mogen worden.

L. van der Pijl, Universiteit en Hogeschool, 17, 464-480 (1971).

VI

In tegenstelling tot een concrete fytocoenose vertoont een vegetatie eenheid geen specifiek minimum areaal.

E. van der Maarel, Utrecht (1966).
A.G.J.M. van der Linden, RIVON Zeist (1967).

VII

Beginnende biologie leraren zouden bij voorkeur in de hóógste klassen van het v.w.o. hun loopbaan moeten starten.

VIII

De huidige geografische verspreiding van Crenobia alpina is volledíger te verklaren uit de wisselwerking tussen milieu en karyotype dan uit de ijstijd relict-hypothese.

A.C. Dahm, Malmö (1958).

IX

De lange dunne spermien nucleus van muggen is indicatief voor tandem positie en niet voor parallelle positie van de chromosomen, zoals Laven en Jost aannemen.

H. Laven and E.Jost,
Experientia 27, 471-473 (1971).

X

Het verband tussen spermien redundantie en de gemiddelde chiasma frequentie per spermatocyt is niet aangetoond.

J. Cohen, Nature, (Lond.) 215, 862-863 (1967).

A.G.J.M. van der Linden
26 november 1971

VOOR MIJN OUDERS